09/849,868

```
=> d his

     (FILE 'HOME' ENTERED AT 14:10:53 ON 05 DEC 2002)

     FILE 'CAPLUS, USPATFULL' ENTERED AT 14:11:50 ON 05 DEC 2002
L1           756 S (HER2 OR HER3)(3A)RECEPTOR?
L2             5 S L1 AND INNER(4A)EAR(P)(CELL? OR GROW? OR GENERAT? OR REGENERA
L3             5 DUP REM L2 (0 DUPLICATES REMOVED)

     FILE 'STNGUIDE' ENTERED AT 14:17:09 ON 05 DEC 2002

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=> d his

     (FILE 'HOME' ENTERED AT 11:00:58 ON 05 DEC 2002)

     FILE 'CAPLUS, USPATFULL' ENTERED AT 11:01:45 ON 05 DEC 2002
L1           976 S HEREGULIN
L2             7 S L1 AND INNER(4A)EAR(P)(CELL# OR GROW? OR GENERAT? OR REGENERA
L3             6 DUP REM L2 (1 DUPLICATE REMOVED)

     FILE 'STNGUIDE' ENTERED AT 11:09:18 ON 05 DEC 2002

     FILE 'CAPLUS, USPATFULL' ENTERED AT 11:13:13 ON 05 DEC 2002
L4             3 S L3 AND (HER2 OR HER3)

     FILE 'STNGUIDE' ENTERED AT 11:21:42 ON 05 DEC 2002

     FILE 'STNGUIDE' ENTERED AT 11:27:25 ON 05 DEC 2002

     FILE 'CAPLUS, USPATFULL' ENTERED AT 11:48:13 ON 05 DEC 2002
L5           996 S (HEREGULIN OR HRG(2A)(ALPHA? OR BETA?) OR RHRG OR RECOMBINAT(
L6             8 S L5 AND INNER(4A)EAR(P)(CELL# OR GROW? OR GENERAT? OR REGENER
L7             7 DUP REM L6 (1 DUPLICATE REMOVED)
```

```
=> s heregulin
L1          976 HEREGULIN

=> s l1 and inner(4a)ear(p)(cell# or grow? or generat? or regenerat? or proliferat?)
L2            7 L1 AND INNER(4A) EAR(P)(CELL# OR GROW? OR GENERAT? OR REGENERAT?
                 OR PROLIFERAT?)

=> dup rem l2
PROCESSING COMPLETED FOR L2
L3            6 DUP REM L2 (1 DUPLICATE REMOVED)

=> d l3 abs ibib kwic 1-6

L3   ANSWER 1 OF 6  USPATFULL
AB     Ligands which bind to the HER2 and/or HER3 receptors are useful as
       inner-ear-supporting cell growth
       factors to enhance proliferation-mediated generation
       of new hair cells.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.
ACCESSION NUMBER:       2002:156704  USPATFULL
TITLE:                  Hair cell disorders
INVENTOR(S):            Gao, Wei-Qiang, Foster City, CA, UNITED STATES

                             NUMBER        KIND    DATE
                        ---------------- ------ --------
PATENT INFORMATION:     US 2002081299      A1   20020627
APPLICATION INFO.:      US 2001-849868     A1   20010504  (9)

                              NUMBER         DATE
                        ------------------ --------
PRIORITY INFORMATION:   US 1998-107522P    19981107 (60)
DOCUMENT TYPE:          Utility
FILE SEGMENT:           APPLICATION
LEGAL REPRESENTATIVE:   KNOBBE MARTENS OLSON & BEAR LLP, 620 NEWPORT CENTER
                        DRIVE, SIXTEENTH FLOOR, NEWPORT BEACH, CA, 92660
NUMBER OF CLAIMS:       18
EXEMPLARY CLAIM:        1
NUMBER OF DRAWINGS:     34 Drawing Page(s)
LINE COUNT:             5225
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB     Ligands which bind to the HER2 and/or HER3 receptors are useful as
       inner-ear-supporting cell growth
       factors to enhance proliferation-mediated generation
       of new hair cells.
SUMM   [0002] This application relates to inducing, promoting, or enhancing the
       growth, proliferation, repair, generation,
       or regeneration of inner ear tissue,
       particularly inner ear epithelial hair cells
       and supporting cells. More particularly, this application
       relates to potently stimulating supporting cell
       proliferation and enhancing proliferation-mediated
       generation of new hair cells. In addition, this
       application provides methods, compositions and devices for prophylactic
       and therapeutic treatment of inner ear disorders and
       conditions, particularly sensorineural hearing and balance impairments.
       This invention relates to the use of HER2 ligands, in particular
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heregulin polypeptides, as **inner-ear** -supporting **cell growth** factors.

SUMM . . . a wide variety of causes, including infections, mechanical injury, loud sounds, aging, and chemical-induced ototoxicity that damages neurons and/or hair **cells** of the peripheral auditory system. The peripheral auditory system consists of auditory receptors, hair **cells** in the organ of Corti, and primary auditory neurons, the spiral ganglion neurons in the cochlea. Spiral ganglion neurons ("SGN") are primary afferent auditory neurons that deliver signals from the peripheral auditory receptors, the hair **cells** in the organ of Corti, to the brain through the cochlear nerve. The eighth nerve connects the primary auditory neurons. . . . are primary afferent sensory neurons responsible for balance and which deliver signals from the utricle, saccule and ampullae of the **inner ear** to the brain, to the brainstem. Destruction of primary afferent neurons in the spiral ganglia and hair **cells** has been attributed as a major cause of hearing impairments. Damage to the peripheral auditory system is responsible for a. . .

SUMM . . . nervous system may result in hearing loss or balance impairment. Auditory apparatus can be divided into the external and middle **ear**, **inner ear** and auditory nerve and central auditory pathways. While having some variations from species to species, the general characterization is common. . . . for all mammals. Auditory stimuli are mechanically transmitted through the external auditory canal, tympanic membrane, and ossicular chain to the **inner ear**. The middle **ear** and mastoid process are normally filled with air. Disorders of the external and middle ear usually produce a conductive hearing. . . . space. Auditory information is transduced from a mechanical signal to a neurally conducted electrical impulse by the action of neuro-epithelial **cells** (hair **cells**) and SGN in the **inner ear**. All central fibers of SGN form synapses in the cochlear nucleus of the pontine brain stem. The auditory projections from. . . . to the auditory brain stem and the auditory cortex. All auditory information is transduced by a limited number of hair **cells**, which are the sensory receptors of the **inner ear**, of which the so-called inner hair **cells**, numbering a comparative few, are critically important, since they form synapses with approximately 90 percent of the primary auditory neurons.. . . . nucleus, the number of neural elements involved is measured in the hundreds of thousands. Thus, damage to a relatively few **cells** in the auditory periphery can lead to substantial hearing loss or balance impairment. Hence, many causes of sensorineural loss can be ascribed to lesions in the **inner ear**. This hearing loss and balance impairment can be progressive. In addition, the hearing becomes significantly less acute because of changes. . . .

SUMM [0007] The toxic effects of these drugs on auditory **cells** and spiral ganglion neurons are often the limiting factor for their therapeutic usefulness. For example, antibacterial aminoglycosides such as gentamicins,. . . . infection has reached advanced stages, but at this point permanent damage may already have been done to the middle and **inner ear** structure. Clearly, ototoxicity is a dose-limiting side-effect of antibiotic administration. For example, nearly 75% of patients given 2 grams of. . .

SUMM [0009] Accordingly, there exists a need for means to prevent, reduce or treat the incidence and/or severity of **inner ear** disorders and hearing impairments involving **inner ear** tissue, particularly **inner ear** hair **cells**,

and optionally, the associated auditory nerves. Of particular interest are those conditions arising as an unwanted side-effect of ototoxic therapeutic. . . a method that provides a safe, effective, and prolonged means for prophylactic or curative treatment of hearing impairments related to **inner ear** tissue damage, loss, or degeneration, particularly ototoxin-induced, and particularly involving **inner ear** hair **cells**. The present invention provides compositions and methods to achieve these goals and others as well.

SUMM [0011] In general an object of the invention is to provide a method of inducing, promoting, or enhancing the **growth**, **proliferation**, repair, or **regeneration** of **inner ear** tissue, particularly **inner ear** hair **cells** and their supporting **cells** for the purpose of promoting repair and healing of inner tissue damage or injury.

SUMM [0012] Accordingly, one object of this invention is to provide a method of treating **inner ear** disorders and conditions in patients, primarily human patients, in need of such treatment. A further object is to provide a method of inducing **inner-ear** -supporting **cell growth**, **generation**, and development, which leads to **generation** of new hair **cells**.

SUMM . . . this invention, it has now been discovered that these objects and the broader objective of treating conditions associated with hair **cell** or **inner-ear**-supporting **cell** damage and injury are achieved by administering to a patient in need of such treatment an effective amount of a **heregulin** ligand, preferably a polypeptide or fragment thereof. These **heregulin** polypeptides, include HRG-.alpha., HRG-.beta.1, HRG-.beta.2, HRG-.beta.3 and other **heregulin** polypeptides which cross-react with antibodies directed against these family members and/or which are substantially homologous as defined below and includes **heregulin** variants such as N-terminal and C-terminal fragments thereof. A preferred **heregulin** is the ligand disclosed in FIG. 1A-1D and further designated HRG-.alpha.. Other preferred heregulins are the ligands disclosed in FIG.. . .

SUMM [0014] In another aspect, the invention provides a method in which **heregulin** agonist antibodies are administered to achieve the objects of the invention. In this embodiment, HER2/HER3 or fragments thereof (which also. . . HER3, preferably Her2. In addition, antibodies may be selected that are capable of binding specifically to individual family members of **heregulin** family, e.g. HRG-.alpha., HRG-.beta.1, HRG-.beta.2, HRG-.beta.3, and which are agonists thereof.

SUMM [0015] In general, the invention is a method of **regenerating** and/or repairing hair **cell** or **inner-ear** -supporting **cell** injury by stimulating **growth** and **proliferation** of **inner-ear**-supporting **cells** to enhance **generation** of new hair **cells** . The hair **cells** may be injured by many types of insults, for example, injury due to surgical incision or resection, chemical or smoke inhalation or aspiration, chemical or biochemical ulceration, **cell** damage due to viral or bacterial infection, etc The **inner-ear**-supporting **cells** which may be affected by the method of the invention include any **inner-ear**-supporting **cell** which expresses HER2 or HER3, preferably Her3. The method of the invention stimulates **growth**

and **proliferation** of the **inner-ear** -supporting **cells** leading to **generation** of new hair **cells** to repair and re-establish the sensorineural contacts in the **inner ear** to allow the affected tissues to develop normal physiological functions more quickly.

SUMM [0016] Accordingly, one embodiment of the invention is a method of inducing **inner-ear**-supporting **cell growth** by contacting a **inner-ear**-supporting **cell** which expresses HER2 receptor with an effective amount of a HER2 activating ligand.

SUMM [0017] A further embodiment is a method of treating **inner ear** hair **cell** injury, caused by ototoxins or acoustic assault for example, by administering to a patient in need thereof an effective amount. . .

DRWD . . . .beta.2-like and .beta.3 in descending order and illustrates the amino acid insertions, deletions, and substitutions that characterize these forms of **heregulin** (SEQ ID NOS: 1, 3, 5, 9, and 7).

DRWD [0027] FIG. 10 shows the dose-dependent proliferation effect of **heregulin** on cells in the rat utricular sheet hair cell layer, as indicated by the number of BrdU positive cells per. . .

DRWD . . . 11A-D show autoradiography of tritiated-thymidine labeled cells in supporting cell and hair cell layer in gentamicin-treated utricles in response to **heregulin** treatment. FIGS. A-D are views from similarly treated organotypic rat utricular whole mounts.

DRWD . . . tritiated-thymidine labeled cells in supporting cell and hair cell layer in gentamicin-treated utricles (shown in FIGS. 11A-D) in response to **heregulin** treatment compared to control.

DRWD [0030] FIG. 13 shows the RNA concentration of **heregulin** and the recepters Her2, Her3 and Her4 in RAN isolated from the inner ear sensory epithelium layer.

DRWD [0031] FIG. 14 shows localization of Her2, a **heregulin** receptor, in the inner ear sensory epithelium, as indicated by immunostaining the P0 cochlea and adult utricle with labeled monoclonal. . .

DETD [0032] **Heregulin** ligands, in particular polypeptides and agonist antibodies thereof, have affinity for and stimulate the HER2, and less preferably HER3, receptors or combinations thereof in autophosphorylation. Included within the definition of **heregulin** ligands, in addition to HRG-.alpha., HRG-.beta.1, HRG-.beta.2, HRG-.beta.3 and HRG-.beta.2-like, are other polypeptides binding to the HER2 receptor, which bear substantial amino acid sequence homology to HRG-.alpha. or HRG-.beta.1. Such additional polypeptides fall within the definition of **heregulin** as a family of polypeptide ligands that bind to the HER2 receptors.

DETD [0033] **Heregulin** polypeptides bind with varying affinities to the HER2 receptors. It is also known that heterodimerization of HER2 with HER3 occurs with subsequent receptor cross-phosphorylation as described above. In the present invention, **inner-ear** -supporting **cell growth** and/or **proliferation** is induced when a **heregulin** protein interacts and binds with an individual receptor molecule or a receptor dimer such that receptor phosphorylation is induced. Binding. . .

DETD . . . superfamily and consists of three distinct receptors, HER2, HER3, and HER4. A ligand for this ErbB family is the protein **heregulin** (HRG), a multidomain containing protein with at least 15 distinct isoforms.

DETD [0039] The quest for the activator of the HER2 oncogene has lead to the

discovery of a family of **heregulin** polypeptides. These proteins appear to result from alternate splicing of a single gene which was mapped to the short arm. . .

DETD [0040] Holmes et al. isolated and cioned a family of polypeptide activators for the HER2 receptor which they called **heregulin** -.alpha. (HRG-.alpha.), **heregulin**-.beta.1 (HRG-.beta.1), **heregulin**-.beta.2 (HRG-.beta.2), **heregulin** -.beta.2-like (HRG-.beta.2-like), and **heregulin**-.beta.3 (HRG-.beta.3). See Holmes et al., Science 256:1205-1210 (1992); WO 92/20798; and U.S. Pat. No. 5,367,060. The 45 kDa polypeptide, HRG-.alpha.,. . . from the conditioned medium of the MDA-MB-231 human breast cancer cell line. These researchers demonstrated the ability of the purified **heregulin** polypeptides to activate tyrosine phosphorylation of the HER2 receptor in MCF7 breast tumor cells. Furthermore, the mitogenic activity of the **heregulin** polypeptides on SK-BR-3 cells (which express high levels of the HER2 receptor) was illustrated. Like other growth factors which belong. . .

DETD [0042] Falls et al., Cell, 72:801-815 (1993) describe another member of the **heregulin** family which they call acetylcholine receptor inducing activity (ARIA) polypeptide. The chicken-derived ARIA polypeptide stimulates synthesis of muscle acetylcholine receptors. See also WO 94/08007. ARIA is a .beta.-type **heregulin** and lacks the entire spacer region rich in glycosylation sites between the Ig-like domain and EGF-like domain of HRG.alpha., and. . .

DETD . . . proteins which they call glial growth factors (GGFs). These GGFs share the Ig-like domain and EGF-like domain with the other **heregulin** proteins described above, but also have an amino-terminal kringle domain. GGFs generally do not have the complete glycosylated spacer region. . .

DETD [0044] Ho et al. in J. Biol. Chem. 270(4):14523-14532 (1995) describe another member of the **heregulin** family called sensory and motor neuron-derived factor (SMDF). This protein has an EGF-like domain characteristic of all other **heregulin** polypeptides but a distinct N-terminal domain. The major structural difference between SMDF and the other **heregulin** polypeptides is the lack in SMDF of the Ig-like domain and the "glyco" spacer characteristic of all the other **heregulin** polypeptides. Another feature of SMDF is the presence of two stretches of hydrophobic amino acids near the N-terminus.

DETD [0045] While the **heregulin** polypeptides were first identified based on their ability to activate the HER2 receptor (see Holmes et al., supra), it was. . . undergo tyrosine phosphorylation (Peles et al., EMBO J. 12:961-971 (1993)). This indicated another cellular component was necessary for conferring full **heregulin** responsiveness. Carraway et al. subsequently demonstrated that .sup.125I-rHRG.beta.1.sub.177-244 bound to NIH-3T3 fibroblasts stably transfected with bovine erbB3 but not to. . . (1994). Sliwkowski et al., J. Biol. Chem. 269(20):14661-14665 (1994) found that cells transfected with HER3 alone show low affinities for **heregulin**, whereas cells transfected with both HER2 and HER3 show higher affinities.

DETD . . . p185.sup.HER4/p185.sup.HER2 activation. They expressed p185.sup.HER2 alone, p185.sup.HER4 alone, or the two receptors together in human T lymphocytes and demonstrated that **heregulin** is capable of stimulating tyrosine phosphorylation of p185.sup.HER4, but could only stimulate p185.sup.HER2 phosphorylation in cells expressing both receptors. Plowman. . .

DETD [0048] The biological role of **heregulin** has been investigated by several groups. For example, Falls et al., (discussed above) found

that ARIA plays a role in. . .

DETD . . . factor for astrocytes (Pinkas-Kramarski et al., PNAS, USA 91:9387-9391 (1994)). Meyer and Birchmeier, PNAS, USA 91:1064-1068 (1994) analyzed expression of **heregulin** during mouse embryogenesis and in the perinatal animal using in situ hybridization and RNase protection experiments. See also Meyer et al., Development 124(18):3575-3586 (1997). These authors conclude that, based on expression of this molecule, **heregulin** plays a role in vivo as a mesenchymal and neuronal factor. Similarly, Danilenko et al., Abstract 3101, FASEB 8(4-5):A535 (1994);. . .

DETD [0057] "**Heregulin**" ligand is defined herein to be any isolated ligand, preferably a polypeptide sequence which possesses a biological property of a naturally occurring **heregulin** polypeptide that binds and activates Her2. Ligands within the scope of this invention include the **heregulin** polypeptides discussed in detail herein. **Heregulin** includes the polypeptides shown in FIGS. 1A-1D, 2A-2E, 3A-3E, 4A-4C, 5A-5D, 6A-6C, and 7A-7C and mammalian analogues thereof. Variants can. . .

DETD [0058] The term a "normal" hair **cell** or **inner-ear**-supporting **cell** means an hair **cell** or **inner-ear**-supporting **cell** which is not transformed, i.e., is non-cancerous and/or non-immortalized. Further, the normal hair **cell** or **inner-ear**-supporting **cell** is preferably not aneuploid. Aneuploidy exists when the nucleus of a **cell** does not contain an exact multiple of the haploid number of chromosomes, one or more chromosomes being present in greater or lesser number than the rest. Typical properties of transformed **cells** which fall outside the scope of this invention include the ability to form tumors when implanted into immune-deprived mice (nude mice), the ability to **grow** in suspension or in semi-solid media such as agar, a loss of contact inhibition allowing piling up of **cells** into colonies or foci, a loss of dependence on **growth** factors or serum, **cell** death if **cells** are inhibited from **growing**, and disorganization of actin filaments. Specifically included within the invention are normal **cells** which will not form tumors in mice, **grow** attached to plastic or glass (are anchorage dependent), exhibit contact inhibition, require serum-containing hormones and **growth** factors, remain viable if **growth** is arrested by lack of serum, and contain well-organized actin filaments. Although the normal **inner-ear**-supporting **cells** are preferably not cultured **cells**, also suitable for the invention are non-transformed, non-immortalized epithelial **cells** isolated from mammalian tissue. These isolated **cells** may be cultured for several **generations** (up to about 10 or even 50 **generations**) in the presence of a **heregulin** in order to induce **growth** and/or **proliferation** of the isolated **inner ear** supporting **cell** sample, that is, to expand the sample. The expanded sample can then be reintroduced into the mammal for the purpose of repopulating the hair **cell** or **inner-ear**-supporting **cell** tissue (re-epithelialization). This is particularly useful for repairing tissue injury or damage.

DETD . . . purposes herein means an in vivo biologic or antigenic function or activity that is directly or indirectly performed by an **heregulin** sequence (whether in its native or denatured conformation), or by any subsequence thereof. Biologic functions include receptor binding, any enzyme. . . i.e. possession of an epitope or

antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring **heregulin** polypeptide.

DETD [0060] "Biologically active" **heregulin** is defined herein as a polypeptide sharing a biologic function of an **heregulin** sequence which may (but need not) in addition possess an antigenic function. A principal known effect or function of **heregulin** is as a ligand polypeptide having a qualitative biological activity of binding to HER2 resulting in the activation of the receptor tyrosine kinase (an "activating ligand"). One test for activating ligands is the **heregulin** tyrosine autophosphorylation assay described below. Included within the scope of **heregulin** as that term is used herein are **heregulin** having translated mature amino acid sequences of the complete human **heregulin** as set forth herein; deglycosylated or unglycosylated derivatives of **heregulin**, amino acid sequence variants of **heregulin** sequence, and derivatives of **heregulin**, which are capable of exhibiting a biological property in common with **heregulin**. While native **heregulin** is a membrane-bound polypeptide, soluble forms, such as those forms lacking a functional transmembrane domain, are also included within this definition. In particular, included are polypeptide fragments of **heregulin** sequence which have an N-terminus at any residue from about S216 to about A227, and its C-terminus at any residue. . .

DETD [0061] "Antigenically active" **heregulin** is defined as a polypeptide that possesses an antigenic function of an **heregulin** and which may (but need not) in addition possess a biologic function.

DETD [0062] In preferred embodiments, antigenically active **heregulin** is a polypeptide that binds with an affinity of at least about 10.sup.-9 I/mole to an antibody raised against a naturally occurring **heregulin** sequence. Ordinarily the polypeptide binds with an affinity of at least about 10.sup.-8 I/mole. Most preferably, the antigenically active **heregulin** is a polypeptide that binds to an antibody raised against one of heregulins in its native conformation. **Heregulin** in its native conformation generally is **heregulin** as found in nature which has not been denatured by chaotropic agents, heat or other treatment that substantially modifies the three dimensional structure of **heregulin** as determined, for example, by migration on nonreducing, nondenaturing sizing gels. Antibody used in this determination may be rabbit polyclonal antibody raised by formulating native **heregulin** from a non-rabbit species in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of anti-**heregulin** antibody plateaus.

DETD [0063] Ordinarily, biologically or antigenically active **heregulin** will have an amino acid sequence having at least 75% amino acid sequence identity with a given **heregulin** sequence, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to an **heregulin** sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with **heregulin** residues in the **heregulin** of FIG. 6A-6C, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not. . . any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into **heregulin** sequence shall be construed as affecting homology.

DETD [0064] Thus, the biologically active and antigenically active

heregulin polypeptides that are the subject of this invention include each entire **heregulin** sequence; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues from **heregulin** sequence; amino acid sequence variants of **heregulin** sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, **heregulin** sequence or its fragment as defined above; amino acid sequence variants of **heregulin** sequence or its fragment as defined above has been substituted by another residue. **heregulin** polypeptides include those containing predetermined mutations by, e.g., site-directed or PCR mutagenesis, and other animal species of **heregulin** polypeptides such as rabbit, rat, porcine, non-human primate, equine, murine, and ovine **heregulin** and alleles or other naturally occurring variants of the foregoing and human sequences; derivatives of **heregulin** or its fragments as defined above wherein **heregulin** or its fragments have been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope); glycosylation variants of **heregulin** (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of appropriate amino acid); and soluble forms of **heregulin**, such as HRG-GFD or those that lack a functional transmembrane domain.

DETD [0065] "Isolated" means a ligand, such as **heregulin**, which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for **heregulin**, and may include proteins, hormones, and other substances. In preferred embodiments, **heregulin** will be purified (1) to greater than 95% by weight of protein as determined by the Lowry method or other. . . marketed on the filing date hereof, or (3) to homogeneity by SDS-PAGE using Coomassie blue or, preferably, silver stain. Isolated **heregulin** includes **heregulin** in situ within recombinant cells since at least one component of **heregulin** natural environment will not be present. Isolated **heregulin** includes **heregulin** from one species in a recombinant cell culture of another species since **heregulin** in such circumstances will be devoid of source polypeptides. Ordinarily, however, isolated **heregulin** will be prepared by at least one purification step.

DETD [0066] In accordance with this invention, **heregulin** nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active **heregulin**, is complementary to nucleic acid sequence encoding such **heregulin** , or hybridizes to nucleic acid sequence encoding such **heregulin** and remains stably bound to it under stringent conditions.

DETD [0067] Preferably, **heregulin** nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with an **heregulin** sequence. Preferably, the **heregulin** nucleic acid that hybridizes contains at least 20, more preferably at least about 40, and most preferably at least about. . .

DETD [0068] Isolated **heregulin** nucleic acid includes a nucleic acid that is identified and separated from at least one containment nucleic acid with which it is ordinarily associated in the natural source of **heregulin** nucleic acid. Isolated **heregulin** nucleic

acid thus is present in other than in the form or setting in which it is found in nature. However, isolated **heregulin** encoding nucleic acid includes **heregulin** nucleic acid in ordinarily **heregulin**-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature. Nucleic acid encoding **heregulin** may be used in specific hybridization assays, particularly those portions of **heregulin** encoding sequence that do not hybridize with other known DNA sequences. "Stringent conditions" are those that (1) employ low ionic. . .

DETD [0081] The "**heregulin** tyrosine autophosphorylation assay" to detect the presence or bioactivity of **heregulin** ligands can be used to monitor the purification of a ligand for the HER2 receptors. This assay is based on. . .

DETD [0097] II. Use and Preparation of **Heregulin** Sequences

DETD [0098] H. Preparation of **Heregulin** Sequences, Including Variants

DETD [0099] The system to be employed in preparing **heregulin** sequence will depend upon the particular **heregulin** sequence selected. If the sequence is sufficiently small **heregulin** may be prepared by in vitro polypeptide synthetic methods. Most commonly, however, **heregulin** will be prepared in recombinant cell culture using the host-vector systems described below. Suitable **heregulin** includes any biologically active and antigenetically active **heregulin**.

DETD . . . In general, mammalian host cells will be employed, and such hosts may or may not contain post-translational systems for processing **heregulin** preprosequences in the normal fashion. If the host cells contain such systems then it will be possible to recover natural. . . vitro method. However, it is not necessary to transform cells with the complete prepro or structural genes for a selected **heregulin** when it is desired to only produce fragments of **heregulin** sequences. For example, a start codon is ligated to the 5' end of DNA encoding an HRG-GFD, this DNA is. . .

DETD [0101] **Heregulin** sequences located between the first N-terminal mature residue and the first N-terminal residue of HRG-GFD sequence, termed HRG-NTD, may function. . . molecule but from expression of DNA in which a stop codon is located at the C-terminus of HRG-NTD. In addition, **heregulin** variants are expressed from DNA encoding protein in which both the GFD and NTD domains are in their proper orientation. . .

DETD [0103] As noted above, other **heregulin** sequences to be prepared in accordance with this invention are those of the GFD. These are synthesized in vitro or. . . in recombinant cell culture. These are produced most inexpensively in yeast or E.coli by secretion under the control of a **heregulin**-heterologous signal as described infra, although preparation in mammalian cells is also contemplated using a mammalian protein signal such as that of tPA, UK or a secreted viral protein. The GFD can be the sequence of a native **heregulin** or may be a variant thereof as described below. GFD sequences include those in which one or more residues from. . .

DETD [0104] An additional **heregulin** is one which contains the GFD and the sequence between the C-terminus of GFD and the N-terminus of the transmembrane. . . C-terminal cleavage domain or CTC). In this variant (HRG-GFD-CTC) the DNA start codon is present at the 5' end of **heregulin**-heterologous signal sequence or adjacent the 5' end of the GFD-encoding region, and a stop codon is found in place of. . .

DETD [0106] If it is desired to prepare the longer **heregulin**

polypeptides and the 5' or 3' ends of the given **heregulin** are not described herein, it may be necessary to prepare nucleic acids in which the missing domains are supplied by homologous regions from more complete **heregulin** nucleic acids. Alternatively, the missing domains can be obtained by probing libraries using the DNAs disclosed in the Figures or. . .

DETD [0107] A. Isolation of DNA Encoding **Heregulin**

DETD [0108] The DNA encoding **heregulin** may be obtained from any cDNA library prepared from tissue believed to possess **heregulin** mRNA and to express it at a detectable level. HRG-.alpha. gene thus may be obtained from a genomic library. Similar procedures may be used for the isolation of other **heregulin**, such as HRG-.beta.1, HRG-.beta.2, or HRG-.beta.3 encoding genes.

DETD . . . preferably human breast, colon, salivary gland, placental, fetal, brain, and carcinoma cell lines. Other biological sources of DNA encoding an **heregulin**-like ligand include other mammals and birds. Among the preferred mammals are members of the following orders: bovine, ovine, equine, murine,. . .

DETD [0118] B. Amino Acid Sequence Variants of **Heregulin**

DETD [0119] Amino acid sequence variants of **heregulin** are prepared by introducing appropriate nucleotide changes into **heregulin** DNA, or by in vitro synthesis of the desired **heregulin** polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for human **heregulin** sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Excluded from the scope of this invention are **heregulin** variants or polypeptide sequences that are not novel and unobvious over the prior art. The amino acid changes also may. . . such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, altering the intra-cellular location of **heregulin** by inserting, deleting, or otherwise affecting the leader sequence of the native **heregulin**, or modifying its susceptibility to proteolytic cleavage.

DETD [0120] The **heregulin** sequence may be proteolytically processed to create a number of **heregulin** fragments. HRG-GFD sequences of HRG-.alpha. all contain the amino acid sequence between HRG-.alpha. cysteine 226 and cysteine 265. The amino. . .

DETD . . . fragment ligands of HRG-.beta.2 based upon the FIG. 3A-3E and HRG-.beta.3 based upon FIG. 4A-4C may be accomplished by cleaving **heregulin** sequences of FIGS. 3A-3E and 4A-4C preferably adjacent to an arginine, lysine, valine or methionine.

DETD [0122] In designing amino acid sequence variants of **heregulin**, the location of the mutation site and the nature of the mutation will depend on **heregulin** characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first. . .

DETD [0123] A useful method for identification of certain residues or regions of **heregulin** polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244:. . . a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the expressed **heregulin** variants are screened for the optimal combination of desired activity.

DETD . . . amino acid sequence variants: the location of the mutation site and the nature of the mutation. These are variants from **heregulin** sequence, and may represent naturally occurring

alleles (which will not require manipulation of **heregulin** DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon **heregulin** characteristic to be modified. Obviously, such variations that, for example, convert **heregulin** into a known receptor ligand, are not included within the scope of this invention, nor are any other **heregulin** variants or polypeptide sequences that are not novel and unobvious over the prior art.

DETD . . . contiguous. Deletions may be introduced into regions of low homology with other EGF family precursors to modify the activity of **heregulin**. Deletions from **heregulin** in areas of substantial homology with other EGF family sequences will be more likely to modify the biological activity of **heregulin** more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of **heregulin** in the affected domain, e.g., cysteine crosslinking, beta-pleated sheet or alpha helix.

DETD . . . or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within **heregulin** sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, and most preferably 1 to 3. Examples of terminal insertions include **heregulin** with an N-terminal methionyl residue (an artifact of the direct expression of **heregulin** in bacterial recombinant cell culture), and fusion of a heterologous N-terminal signal sequence to the N-terminus of **heregulin** to facilitate the secretion of mature **heregulin** from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host. . .

DETD [0127] Other insertional variants of **heregulin** include the fusion to the N- or C-terminus of **heregulin** of an immunogenic polypeptide, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions of **heregulin**-ECD with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described. . .

DETD [0128] Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in **heregulin** molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of **heregulin**, and sites where the amino acids found in **heregulin** ligands from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. A likely sub-domain of HRG-GFD. . .

DETD [0129] Other sites of interest are those in which particular residues of **heregulin**-like ligands obtained from various species are identical. These positions may be important for the biological activity of **heregulin**. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a. . .

DETD [0130] Substantial modifications in function or immunological identity of **heregulin** are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone. . .

DETD . . . entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of

heregulin that are homologous with other receptor ligands, or, more preferably, into the non-homologous regions of the molecule.

DETD [0140] Any cysteine residues not involved in maintaining the proper conformation of **heregulin** also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DETD [0152] Another **heregulin** variant is or gamma-**heregulin** . -HRG is any polypeptide sequence that possesses at least one biological property of native sequence -HRG having SEQ ID NO:11. The biological property of this variant is the same as for **heregulin** noted above. This variant encompasses not only the polypeptide isolated from a native -HRG source such as human MDA-MB-175 cells. . . residues within the amino acid sequence shown for the human protein in FIG. 7A-7C as generally described agove for other **heregulin**. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct. . .

DETD . . . the variant has an amino acid substitution at a selected residue corresponding to a residue of 645-amino acid native human **heregulin**-.beta.1 selected from:

DETD [0156] Other **heregulin**-.beta.1 variants include an amino acid substitution selected from:

DETD [0158] In a variation of this embodiment, the **heregulin** variant includes sets of amino acid substitutions selected from this group.

DETD [0159] In addition to including one or more of the amino acid substitutions disclosed herein, the **heregulin** variant can have one or more other modifications, such as an amino acid substitution, an insertion of at least one. . . amino acid, a deletion of at least one amino acid, or a chemical modification. For example, the invention provides a **heregulin** variant that is a fragment. In a variation of this embodiment, the fragment includes residues corresponding to a portion of human **heregulin**-.beta.1 extending from about residue 175 to about residue 230 (i.e., the EGF-like domain). For example, the fragment can extend from. . .

DETD [0160] DNA encoding amino acid sequence variants of **heregulin** is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation. . . by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of **heregulin**. These techniques may utilize **heregulin** nucleic acid (DNA or RNA), or nucleic acid complementary to **heregulin** nucleic acid.

DETD [0161] Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of **heregulin** DNA. This technique is well known in the art as described by Adelman et al., DNA, 2: 183 (1983). Briefly, **heregulin** DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of **heregulin**. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in **heregulin** DNA.

DETD . . . DNA encodes the mutated form of heregutin, and the other strand (the original template) encodes the native, unaltered sequence of **heregulin**. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as E. coli JM101. After.

. .

DETD [0166] DNA encoding **heregulin** mutants with more than one amino acid to be substituted may be generated in one of several ways. If the. . .

DETD [0169] PCR mutagenesis is also suitable for making amino acid variants of **heregulin**. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR. . .

DETD . . . technique described by Wells et al. (Gene, 34: 315, 1985). The starting material is the plasmid (or other vector) comprising **heregulin** DNA to be mutated. The codon(s) in **heregulin** DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified. . . restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in **heregulin** DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize. . . of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated **heregulin** DNA sequence.

DETD [0180] The cDNA or genomic DNA encoding native or variant **heregulin** is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available,. . .

DETD [0182] In general, the signal sequence may be a component of the vector, or it may be a part of **heregulin** DNA that is inserted into the vector. The native **heregulin** DNA is believed to encode a signal sequence at the amino terminus (5' end of the DNA encoding **heregulin**) of the polypeptide that is cleaved during post-translational processing of the polypeptide to form the mature **heregulin** polypeptide ligand that binds to the HER2/HER3 receptor, although a conventional signal structure is not apparent. Native **heregulin** is, secreted from the cell but remains lodged in the membrane because it contains a transmembrane domain and a cytoplasmic region in the carboxyl terminal region of the polypeptide. Thus, in a secreted, soluble version of **heregulin** the carboxyl terminal domain of the molecule, including the transmembrane domain, is ordinarily deleted. This truncated variant **heregulin** polypeptide may be secreted from the cell, provided that the DNA encoding the truncated variant encodes a signal sequence recognized. . .

DETD [0183] **Heregulin** of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a. . . polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of **heregulin** DNA that is inserted into the vector. Included within the scope of this invention are **heregulin** with the native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be. . . by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native **heregulin** signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II leaders. For yeast secretion the native **heregulin** signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the. . .

DETD . . . in Bacillus genomic DNA. Transfection of Bacillus with this

vector results in homologous recombination with the genome and insertion of **heregulin** DNA. However, the recovery of genomic DNA encoding **heregulin** is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise **heregulin** DNA. DNA can be amplified by PCR and directly transfected into the host cells without any replication component.

DETD . . . example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up **heregulin** nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which. . . in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes **heregulin**. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of **heregulin** are synthesized from the amplified DNA.

DETD . . . of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding **heregulin**. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of. . . employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding **heregulin**, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in. . .

DETD . . . Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to **heregulin** nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as **heregulin** to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters. . . promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding **heregulin** by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native **heregulin** promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of **heregulin** DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed **heregulin** as compared to the native **heregulin** promoter.

DETD . . . are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding **heregulin** (Siebenlist et al., Cell 20: 269, 1980) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding **heregulin**.

DETD [0200] **Heregulin** gene transcription from vectors in mammalian host cells may be controlled by promoters obtained from the genomes of viruses such. . . mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with **heregulin** sequence, provided such promoters are compatible with the host cell systems.

DETD [0203] Transcription of a DNA encoding **heregulin** of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting. . . . for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to **heregulin** DNA, but is preferably located at a site 5' from the promoter.

DETD . . . DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding **heregulin**. The 3' untranslated regions also include transcription termination sites.

DETD . . . the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding **heregulin**. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host. . . . physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of **heregulin** that have **heregulin**-like activity. Such a transient expression system is described in U.S. Pat. No. 5,024,939.

DETD [0209] Other methods, vectors, and host cells suitable for adaptation to the synthesis of **heregulin** in recombinant vertebrate cell culture are described in Gething et al., Nature 293: 620-625, 1981; Mantei et al., Nature, 281:. . . 1979; Levinson et al., EP 117,060 and EP 117,058. A particularly useful expression plasmid for mammalian cell culture expression of **heregulin** is pRK5 (EP pub. no. 307,247) or pSVI6B (U.S. Ser. No. 07/441,574, filed Nov. 22, 1989, the disclosure of which. . .

DETD [0212] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for **heregulin** -encoding vectors. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number. . .

DETD [0213] Suitable host cells for the expression of glycosylated **heregulin** polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any. . . cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens, which has been previously manipulated to contain **heregulin** DNA. During incubation of the plant cell culture with A. tumefaciens, the DNA encoding **heregulin** is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express **heregulin** DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and. . .

DETD [0219] Prokaryotic cells used to produce **heregulin** polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

DETD [0220] The mammalian host cells used to produce **heregulin** of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal. . .

DETD [0222] It is further envisioned that **heregulin** of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding **heregulin** currently in use in the field. For example, a powerful promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element. . . genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired

heregulin. The control element does not encode **heregulin** of this invention, but the DNA is present in the host cell genome. One next screens for cells making **heregulin** of this invention, or increased or decreased levels of expression, as desired.

DETD . . . either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native **heregulin** polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further below.

DETD [0227] G. Purification of The **Heregulin** Polypeptides

DETD [0228] **Heregulin** is recovered from a cellular membrane fraction. Alternatively, a proteolytically cleaved or a truncated expressed soluble **heregulin** fragment or subdomain are recovered from the culture medium as a soluble polypeptide. A **heregulin** is recovered from host cell lysates when directly expressed without a secretory signal.

DETD [0229] When **heregulin** is expressed in a recombinant cell other than one of human origin, **heregulin** is completely free of proteins or polypeptides of human origin. However, it is desirable to purify **heregulin** from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to **heregulin**. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. **Heregulin** is then be purified from both the soluble protein fraction (requiring the presence of a protease) and from the membrane fraction of the culture lysate, depending on whether **heregulin** is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation;. . .

DETD [0230] **Heregulin** variants in which residues have been deleted, inserted or substituted are recovered in the same fashion as the native **heregulin**, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a **heregulin** fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-**heregulin** column can be employed to absorb **heregulin** variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenylmethylsulfonylfluoride (PMSF) also may. . . to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native **heregulin** may require modification to account for changes in the character of **heregulin** variants or upon expression in recombinant cell culture.

DETD [0231] H. Covalent Modifications of **Heregulin**

DETD [0232] Covalent modifications of **heregulin** polypeptides are included within the scope of this invention. Both native **heregulin** and amino acid sequence variants of **heregulin** optionally are covalently modified. One type of covalent modification included within the scope of this invention is a **heregulin** polypeptide fragment. **Heregulin** fragments, such as HRG-GDF, having up to about 40 amino acid residues are conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length **heregulin** polypeptide or **heregulin** variant polypeptide. Other types of covalent modifications of **heregulin** or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of **heregulin** or

fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N-. . .

DETD [0239] Derivatization with bifunctional agents is useful for crosslinking **heregulin** to a water-insoluble support matrix or surface for use in a method for purifying anti-**heregulin** antibodies, and vice versa, Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid,. . .

DETD [0242] **Heregulin** optionally is fused with a polypeptide heterologous to **heregulin**. The heterologous polypeptide optionally is an anchor sequence such as that found in a phage coat protein such as M13 gene III or gene VIII proteins. These heterologous polypeptides can be covalently coupled to **heregulin** polypeptide through side chains or through the terminal residues.

DETD [0243] **Heregulin** may also be covalently modified by altering its native glycosylation pattern. One or more carbohydrate substitutents in these embodiments, are modified by adding, removing or varying the monosaccharide components at a given site, or by modifying residues in **heregulin** as that glycosylation sites are added or deleted.

DETD [0245] Glycosylation sites are added to **heregulin** by altering its amino acid sequence to contain one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites).. . . alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to **heregulin** (for O-linked glycosylation sites). For ease, **heregulin** is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding **heregulin** at preselected bases such that codons are generated that will translate into the desired amino acids.

DETD [0246] Chemical or enzymatic coupling of glycosides to **heregulin** increases the number of carbohydrate substituents. These procedures are advantageous in that they do not require production of the polypeptide. . .

DETD [0247] Carbohydrate moieties present on an **heregulin** also are removed chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an. . . al. (Arch. Biochem. Biophys., 259:52 (1987)) and by Edge et al. (Anal. Biochem., 118:131 (1981)). Carbohydrate moieties are removed from **heregulin** by a variety of endo- and exo-glycosidases as described by Thotakura et al. (Meth. Enzymol., 138:350 (1987)).

DETD [0249] **Heregulin** may also be modified by linking **heregulin** to various nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos.. . .

DETD [0250] One preferred way to increase the in vivo circulating half life of non-membrane bound **heregulin** is to conjugate it to a polymer that confers extended half-life, such as polyethylene glycol (PEG). (Maxfield, et al, Polymer. . .

DETD [0251] **Heregulin** may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or. . .

DETD . . . in order to select the optimal variant for the purpose intended. For example, a change in the immunological character of **heregulin**, such as a change in affinity for a given antigen or for the HER2 receptor, is measured by a competitive-type immunoassay using a standard or control such as a native **heregulin** (in

particular native **heregulin**-GFD). Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability. . .

DETD . . . or by FACS using celi-bound receptor and labeled candidate antibody. Agonist antibodies are preferably antibodies which stimulate autophosphorylation in the **heregulin** tyrosine autophosphorylation assay described above.

DETD [0271] The **heregulin** are used in the present invention to induce **inner-ear**-supporting **cell proliferation** to enhance new hair **cell generation**. These effects allow treatment of disease states associated with tissue damage, for example, ototoxic injury, or acoustic assault, degenerative hearing. . .

DETD . . . The field of cochlear implantation has also provided insights into both the short- and long-term effects of cochlear fenestration on **inner ear** function. Administration of **growth** factors to the inner ears of animals is now possible with the use of implanted catheters and miniature infusion pumps. Localized application of **heregulin** to the human **inner ear** can be performed to treat **inner ear** disorders related to hair **cell** disfunction.

DETD [0273] Therapeutic formulations of **heregulin** or agonist antibody are prepared for storage by mixing the **heregulin** protein having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, supra), in. . .

DETD [0274] **Heregulin** or agonist antibody to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The **heregulin** or antibody ordinarily will be stored in lyophilized form or in solution.

DETD [0275] Therapeutic **heregulin** or antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag. . .

DETD [0276] The route of **heregulin** or antibody administration is in accord with known methods, e.g., injection or infusion administration to the inner ear, or intralesional routes, or by sustained release systems as noted below. The **heregulin** ligand may be administered continuously by infusion or by bolus injection. An agonist antibody is preferably administered in the same. . .

DETD [0277] The **heregulin**, **heregulin** variant or fragment and agonist antibodies may be spray dried or spray freeze dried using known techniques (Yeo et al,. . .

DETD [0279] Sustained-release **heregulin** or antibody compositions also include liposomally entrapped **heregulin** or antibody. Liposomes containing **heregulin** or antibody are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Nat. Acad. Sci. USA, 82:. . . which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal **heregulin** therapy. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

DETD . . . of infection of a mammal by administration of an aminoglycoside antibiotic, the improvement comprising administering a therapeutically effective amount of **heregulin** or agonist, to the patient in need of such treatment to reduce or prevent ototoxin-induced hearing impairment associated with the. . .

DETD [0282] Also provided herein are methods for promoting new **inner**

ear hair **cells** by inducing **inner ear** supporting **cell proliferation**, **regeneration**, or **growth** upon, prior to, or after exposure to an agent or effect that is capable of inducing a hearing or balance. . . impairment or disorder. Such agents and effects are those described herein. The method includes the step of administering to the **inner ear** hair **cell** an effective amount of **heregulin** or agonist or factor disclosed herein as useful. Preferably, the method is used upon, prior to, or after exposure to. . .

DETD [0287] The **heregulin** or agonist is directly administered to the patient by any suitable technique, including parenterally, intranasally, intrapulmonary, orally, or by absorption. . .

DETD [0288] The **heregulin** or antibody agonist, can be combined and directly administered to the mammal by any suitable technique, including infusion and injection.. . . of administration will depend, e.g., on the medical history of the patient, including any perceived or anticipated side effects using **heregulin** alone, and the particular disorder to be corrected. Examples of parenteral administration include subcutaneous, intramuscular, intravenous, intraarterial, and intraperitoneal administration.. . .

DETD [0290] An effective amount of **heregulin** or antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Also, the amount of **heregulin** polypeptide will generally be less than the amount of an agonist antibody. Accordingly, it will be necessary for the therapist. . . 1 mg/kg and up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer **heregulin** or antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored. . .

DETD [0291] In a further embodiment, **inner-ear**-supporting **cells** may be obtained or isolated from a mammalian tissue to obtain a normal **inner-ear**-supporting **cell** sample using techniques well known in the art (biopsy, etc.). This sample may then be treated with a **heregulin** protein in order to induce hair **cell** or **inner-ear**-supporting **cell growth** and/or **proliferation** in the sample thereby expanding the population of **inner-ear**-supporting **cells**. Typically, **heregulin** will be added to the in vitro **inner-ear**-supporting **cell** culture at a concentration of about 0.1 to about 100 nM preferably 1-50 nM. If desired, the primary **inner-ear**-supporting **cells** may be cultured in vitro for several **generations** in order to sufficiently expand the hair **cell** or **inner-ear**-supporting **cell** population. The hair **cell** or **inner-ear**-supporting **cells** are cultured under conditions suitable for mammalian **cell** culture as discussed above. After expansion, the expanded sample is reintroduced into the mammal for the purpose of re-epithelializing the. . .

DETD [0292] The methods and procedures described herein with respect to HRG-.alpha. or **heregulin** in general may be applied similarly to other **heregulin** such as HRG-.beta.1, HRG-.beta.2 and HRG-.beta.3 and to variants thereof, as well as to the antibodies. All references cited in. . .

DETD [0293] Characterization of **Inner-Ear**-Supporting **Cell** Cultures

DETD [0299] A much greater number of BrdU-positive cells were seen in the cultures containing **heregulin** (HRG-.beta.1-177-244) than any of the other factors known to activate Her receptors. Cell counts performed from the control cultures and cultures containing confirmed that **heregulin** significantly enhanced proliferation of the utricular supporting cells ($p<0.0001$, FIG. 9). IGF-1 at 100 nM, TGF-.alpha. at 100 nM (R. . . et al., EMBO Journal 16(6):1268-78 (1997)), and IGF1-binding protein at 100 nM were weaker mitogens, if at all, compared to **heregulin**. SMDF polypeptides are prepared as described in WO 96/15244. Neuregulin-3, a neural tissue-enriched protein that binds and activates erbB4, was. . .

DETD [0300] To determine whether the effect of **heregulin** was dose-dependent, a dose-dependent study was carried out in the utricular epithelial sheet cultures at a range of 0.03 nM to 10 nM **heregulin** (FIG. 10). A **heregulin**-dose-dependent increase in the number of BrdU positive cells was observed. Maximal effect of **heregulin** was seen at 3 nM.

DETD . . . in Zheng et al. (Journal of Neuroscience, 17(21):8270-82 (1997)). This system provides an excellent means to test the effect of **heregulin** on supporting cell proliferation in a physiologically significant system that mimics the in vivo state. In particular, the effects of **heregulin** after ototoxic-induced damage (e.g. antibiotic gentamycin) were examined.

DETD . . . mounts were cultured 1-2 days after explant, then treated with gentamycin (1 mM) for two days, and then treated with **heregulin** (3 nM) for 11 days in the presence of tritiated thymidine. To determine the number of labeled **cells**, the tissue was fixed, sectioned and processed for autoradiography. In response to **heregulin**, compared to control cultures, an increase in the number of .sup.3H-thymidine labeled **cells** in both the supporting **cell** layer (SC) and the hair **cell** layer (HC) was observed as shown in FIGS. 11A-D, which represent similarly treated samples. The **cell** count of .sup.3H-thymidine labeled **cells** in both the supporting **cell** layer and in the hair **cell** layer increased significantly compared to control cultures lacking **heregulin** as shown in FIG. 12. The data is consistent with the data obtained in the utricular sheet cultures. And the data indicates that **heregulin** can act to increase **inner-ear**-supporting **cell** **proliferation**, which leads to hair **cell** **generation**, in instances following hair **cell** damage and injury.

DETD [0306] **Heregulin** Acts through the Her2 Receptor

DETD [0307] To provide further evidence that **heregulin** is a physiologically relevant factor and that it acts through a physiologically relevant receptor, the mRNA expression levels of **heregulin** and its receptors Her2, Her3 and Her4 in the hair **cell** and supporting **cell** layers of the rat utricular sensory epithelium were determined. RNA was extracted from the P3 utricle sheet cultures and also from UEC4 **cells** (a **inner-ear**-supporting **cell** line). Using TaqMan PCR analysis with appropriate gene-specific primers (Heid et al., Genome Research. 6(10):986-94 (1996)), it was observed that all four were expressed in the **inner ear**, however, **heregulin** and Her2 were expressed at a higher level than either Her3 or Her4 (see FIG. 13). Her4 was not expressed in the **inner**-**ear**-supporting **cell** line.

DETD . . . monoclonal antibody was used to immunostain rat P0 (day zero)

cochlea and adult utricle. Her2 was localized to the hair **cell** and supporting **cell** sensory epithelium layers in the **inner ear** (see FIG. 14 A (cochlea) and FIG. 14B (utricle)). Anti-HER2 monoclonal antibodies 2C4 and 4D5 have been described elsewhere (Fendly et al. Cancer Research 50:1550-1558 (1990)). Consistent with this observation is that immunostaining with a **heregulin** antibody suggests that **heregulin** is expressed by hair **cells** of the **inner ear**.

DETD . . . Her2, but not the addition of the immunoadhesin Her4-IgG, at saturating amounts to the utricular cultures, blocked the effects of **heregulin**. Thus, **heregulin** stimulates supporting cell proliferation and hence the generation of new hair cells by activating a Her2-mediated signaling pathway, but not. . .

DETD [0310] In addition, preliminary experiments with embryonic rat **inner ear** explant cultures show that **heregulin** affects hair **cell** differentiation by enhancing **proliferation** of hair **cell** progenitors. Rat E14 otocyst cultures treated with **heregulin** respond with an increase in the number of hair **cell** progenitor **cells** compared to untreated cultures. This is consistent with the adult tissue studies, indicating that **heregulin** stimulates the **proliferation** of **cells** that differentiate into hair **cells**.

DETD [0311] **Heregulin** Acts In Vivo to Enhance **Inner Ear** Supporting **Cell Proliferation** and Hair **Cell Generation** Following Ototoxic Injury and Acoustic Assault

DETD [0312] Chinchillas are an accepted model to test the effects of factors and agents against or following hair **cell** damage or injury. Chinchillas can be treated with gentamicin, caboplatin or acoustic trauma. Preferably, at least five chinchillas are in. . . assault and allowed to recover. Typically, four to six weeks is sufficient for recovery. The test group is treated with **heregulin** in addition to the injury. All animals will receive BrdU, preferably subcutaneous infusion, using minipumps, to label the dividing **cells** during the treatment period. **Heregulin**, or one of the **heregulin** factors as taught herein, will be administered to the **inner ear**. Minipumps can be used. The **heregulin** can be infused into the cochlea. After the treatment period, cochlea and utricular maculae are dissected out of the animals. The tissue is fixed and BrdU immunohistochemical labeling done. BrdU labeled **cells** in the **inner ear** sensory epithelium are counted. **Cell** counts from the two groups--are compared and analyzed statistically to determine the amount of enhancement of **proliferation** of supporting **cells** and new hair **cell generation** induced by the **heregulin** treatment.

DETD [0532] Forge A, Li L, Corwin J T, Nevill G (1993) Ultrastructural evidence for hair **cell regeneration** in the mammalian **inner ear**. Science 259:1616-1619.

DETD [0568] Lambert P R (1994) **Inner ear** hair **cell regeneration** in a mammal: identification of a triggering factor. Laryngoscope 104:701-718.

DETD [0606] Tsue T T, Oesterle E C, Rubel E W (1994a) Diffusible factors regulate hair **cell regeneration** in the avian **inner ear**. Proc Natl Acad. Sci USA 91:1584-1588.

DETD [0607] Tsue T T, Oesterle E C, Rubel E W (1994b) Hair **cell regeneration** in the **inner ear**. Otolaryngol.

Head Neck Surg 111:281-301.

DETD . . . S., Patterson, S. L., Heuer, J. G., Wheeler, E. F., Bothwell, M., and Rubel, E. W. (1991). Expression of nerve **growth** factor (NGF) receptors in the developing **inner ear** of chick and rat. Development 113: 455-470.

DETD [0612] Warchol, M. E., Lambert, P. R., Goldstein, B. J., Forge, A., and Corwin, J. T. (1993). **Regenerative proliferation** in **inner ear** sensory epithelia from adult Guinea pigs and humans. Science 259:1619-1622.

DETD [0618] Yamashita H, Oesterle E C (1995) Induction of **cell proliferation** in mammalian **inner-ear** sensory epithelia by transforing **growth** factor a and epidermal **growth** factor. Proc Natl Acad Sci USA 92:3152-3155.

CLM What is claimed is:

1. A method of inducing hair **cell generation** or **inner-ear**-supporting **cell growth**, **regeneration**, and/or **proliferation**, comprising contacting an **inner-ear**-supporting **cell** which expresses HER2 and/or HER3 receptors with an effective amount of an isolated ligand which activates HER2 and/or HER3 receptors. . .

2. The method of claim 1, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the HER2 or HER3 receptor.

3. The method of claim 2, wherein the activating ligand is human **heregulin** or a fragment thereof.

6. The method of claim 2, wherein the activating ligand is recombinant human **heregulin** or a fragment thereof.

11. The method of claim 6, wherein the **heregulin** is rHRG-.beta.1-177-244.

12. The method of claim 1, wherein the **inner-ear** -supporting **cell** is in the utricle or cochlea.

13. The method of claim 1 wherein the **inner-ear** -supporting **cell** expresses HER2, HER3, or both.

14. A method of increasing the number of **inner ear** supporting **cells**, comprising administering to a patient in need thereof an effective amount of an isolated HER2 and/or HER3 activating ligand.

15. The method of claim 14, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the HER2 and/or HER3 receptor.

17. The method of claim 16, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the HER2 and/or HER3 receptor.

18. A method, comprising the steps of: (a) obtaining an **inner** -**ear**-supporting **cell** sample from a mammal; (b) contacting the sample with a ligand which activates HER2 or HER3 or a

combination thereof to induce **growth** and/or **proliferation** of **inner-ear**-supporting **cells** in the sample and to obtain an expanded sample; and (c) re-introducing the expanded sample into the mammal.

L3 ANSWER 2 OF 6 CAPLUS COPYRIGHT 2002 ACS DUPLICATE 1

AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. The peptide of the invention is SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCQNYVMAS. A deriv. of the peptide with polyethylene glycol, dextran or a polyamino acid can also be used. The peptides are expected to be useful to treat vestibular disorders such as, for example, loss of balance, and to treat hearing loss.

ACCESSION NUMBER: 2000:67485 CAPLUS
DOCUMENT NUMBER: 132:88182
TITLE: Use of NDF peptide as **growth** factor for sensory epithelium of the **inner ear**
INVENTOR(S): Carnahan, Josette F.
PATENT ASSIGNEE(S): Amgen Inc., USA
SOURCE: U.S., 11 pp., Cont. of U.S. Ser. No. 129,549, abandoned.
CODEN: USXXAM
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 2
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 6017886	A	20000125	US 1999-255974	19990223

PRIORITY APPLN. INFO.: US 1998-129549 B1 19980805
REFERENCE COUNT: 17 THERE ARE 17 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI Use of NDF peptide as **growth** factor for sensory epithelium of the **inner ear**

AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. The peptide of the invention is SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCQNYVMAS. A deriv. of the peptide with polyethylene glycol, dextran or a polyamino acid can also be used. The peptides are expected to be useful to treat vestibular disorders such as, for example, loss of balance, and to treat hearing loss.

ST NDF peptide **growth** factor sensory epithelium **inner ear**

IT **Ear**
(**inner**; use of NDF peptide as **growth** factor for sensory epithelium for treatment of vestibular disorders and hearing loss)

L3 ANSWER 3 OF 6 CAPLUS COPYRIGHT 2002 ACS

AB Ligands which bind to the HER2 and/or HER3 receptors are useful as **inner-ear**-supporting **cell-growth** factors to enhance **proliferation**-mediated **generation**

of new hair **cells**, e.g. in treatment of hearing disorders. Thus, in cultures of rat utricular epithelial sheets, **heregulin** HRG-.beta.1-177-244 significantly enhanced **proliferation** of utricular supporting **cells**. In chinchillas, **heregulin** acts in vivo to enhance **inner ear** supporting **cell proliferation** and hair **cell generation** following ototoxic injury and acoustic assault. Heregulins may also be used ex vivo for expansion of supporting **cells**, followed by reimplantation into the **inner ear**.

ACCESSION NUMBER: 2000:335267 CAPLUS
DOCUMENT NUMBER: 133:814
TITLE: Method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors
INVENTOR(S): Gao, Wei-qiang
PATENT ASSIGNEE(S): Genentech, Inc., USA
SOURCE: PCT Int. Appl., 141 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000027426	A1	20000518	WO 1999-US25744	19991028
W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
CA 2345899	AA	20000518	CA 1999-2345899	19991028
EP 1126873	A1	20010829	EP 1999-956853	19991028
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO				
US 2002081299	A1	20020627	US 2001-849868	20010504

PRIORITY APPLN. INFO.: US 1998-107522P P 19981107
WO 1999-US25744 W 19991028

REFERENCE COUNT: 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI Method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors

AB Ligands which bind to the HER2 and/or HER3 receptors are useful as **inner-ear**-supporting **cell-growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**, e.g. in treatment of hearing disorders. Thus, in cultures of rat utricular epithelial sheets, **heregulin** HRG-.beta.1-177-244 significantly enhanced **proliferation** of utricular supporting **cells**. In chinchillas, **heregulin** acts in vivo to enhance **inner ear** supporting **cell proliferation** and hair **cell generation** following ototoxic injury and acoustic assault.

Heregulins may also be used ex vivo for expansion of supporting **cells**, followed by reimplantation into the **inner ear**.

ST ear hair **cell regeneration heregulin**; HER receptor ligand **inner ear**; **cell proliferation inner ear heregulin**

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.alpha.; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.1; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.2-like; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.2; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.3; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Ear
(cochlea, implant; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Ear**
(disease; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Animal tissue culture
Molecular cloning
(enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(enhancing **proliferation** of **inner ear**

hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Growth** factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-3, ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Growth** factor receptors
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(**heregulin**, erbB-3, ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Antibodies
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**heregulin**-agonistic; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Drug delivery systems
(implants, cochlear; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Ear**
(**inner**, supporting **cell**; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Ear**
(**inner**, utricle; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT neu (receptor)
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Ear
(organ of Corti, hair **cell**; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(.gamma.-HRG; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT 142158-51-8 142158-52-9 142158-53-0 146591-70-0 146591-82-4 178862-39-0 196678-45-2 270245-15-3 270245-16-4, 14: PN: WO0027426 SEQID: 2 unclaimed DNA 270245-18-6
RL: PRP (Properties)
(unclaimed nucleotide sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors)

IT 146591-75-5, **Heregulin** .beta.2 (human clone .lambda.her76 precursor reduced) 146591-80-2, Protein (human clone .lambda.her84 **heregulin** .beta.2-like precursor reduced) 168183-94-6

198086-50-9, **Heregulin** (human gene .gamma.-HRG) 270245-14-2
270245-17-5
RL: PRP (Properties)
(unclaimed protein sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors)

IT 146591-69-7, 1-625-**Heregulin** .alpha. (human clone .lambda.gt10her16 precursor reduced) 146591-71-1 146591-78-8, **Heregulin** .beta.3 (human clone .lambda.her78 precursor reduced) 260348-98-9 270560-38-8 270560-39-9 270560-40-2
RL: PRP (Properties)
(unclaimed sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors)

L3 ANSWER 4 OF 6 USPATFULL

AB Compositions, methods, and devices are provided for inducing or enhancing the **growth**, **proliferation**, **regeneration** of **inner ear** tissue, particularly **inner ear** hair **cells**. In addition, provided are compositions and methods for prophylactic or therapeutic treatment of a mammal afflicted with an **inner ear** disorder or condition, particularly for hearing impairments involving hair **cell** damage, loss, or degeneration, by administration of a therapeutically effective amount of IGF-1 or FGF-2, or their agonists, alone or in combination.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

ACCESSION NUMBER:	2000:164484 USPATFULL		
TITLE:	Treatment of **inner ear** hair **cells**		
INVENTOR(S):	Gao, Wei-Qiang, Foster City, CA, United States		
PATENT ASSIGNEE(S):	Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)		
	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6156728		20001205
APPLICATION INFO.:	US 1997-963596		19971031 (8)
	NUMBER		DATE
PRIORITY INFORMATION:	US 1996-29536P		19961101 (60)
	US 1996-30278P		19961104 (60)
DOCUMENT TYPE:	Utility		
FILE SEGMENT:	Granted		
PRIMARY EXAMINER:	Moezie, F. T.		
LEGAL REPRESENTATIVE:	Knobbe Martens Olson & Bear, LLP.		
NUMBER OF CLAIMS:	11		
EXEMPLARY CLAIM:	1		
NUMBER OF DRAWINGS:	17 Drawing Figure(s); 7 Drawing Page(s)		
LINE COUNT:	2344		

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI Treatment of **inner ear** hair **cells**

AB Compositions, methods, and devices are provided for inducing or enhancing the **growth**, **proliferation**, **regeneration** of **inner ear** tissue, particularly **inner ear** hair **cells**. In

addition, provided are compositions and methods for prophylactic or therapeutic treatment of a mammal afflicted with an **inner ear** disorder or condition, particularly for hearing impairments involving hair **cell** damage, loss, or degeneration, by administration of a therapeutically effective amount of IGF-1 or FGF-2, or their agonists, alone or. . .

SUMM This application relates to inducing, promoting, or enhancingthe **growth**, **proliferation**, or **regeneration** of **inner ear** tissue, particularly **inner ear** epithelial hair **cells**. In addition, this application provides methods, compositions and devices for prophylactic and therapeutic treatment of **inner ear** disorders and conditions, particularly hearing impairments. The methods comprise administration of insulin-like **growth** factor-I (IGF-1) and/or fibroblast **growth** factor-2 (FGF-2), or their agonists.

SUMM . . . a wide variety of causes, including infections, mechanical injury, loud sounds, aging, and chemical-induced ototoxicity that damages neurons and/or hair **cells** of the peripheral auditory system. The peripheral auditory system consistsof auditoryreceptors,hair **cells** in the organ of Corti, and primary auditory neurons, the spiral ganglion neurons in the cochlea. Spiral ganglion neurons ("SGN") are primary afferent auditory neurons that deliver signals from the peripheral auditory receptors, the hair **cells** in the organ of Corti, to the brain through the cochlear nerve. The eighth nerve connects the primary auditory neurons. . . are primary afferent sensory neurons responsible for balance and which deliver signals from the utricle, saccule and ampullae of the **inner ear** to the brain, to the brainstem. Destruction of primary afferent neurons in the spiral ganglia and hair **cells** has been attributed as a major cause of hearing impairments Damage to the peripheral auditory system is responsible for a. . .

SUMM . . . to the central nervous system may result in hearing loss. Auditory apparatus can be divided into the external and middle **ear**, **inner ear** and auditory nerve and central auditory pathways. While having some variations from species to species, the general characterization is common. . . for all mammals. Auditory stimuli are mechanically transmitted through the external auditory canal, tympanic membrane, and ossicular chain to the **inner ear**. The middle **ear** and mastoid process are normally filled with air. Disorders of the external and middle ear usually produce a conductive hearing. . . space. Auditory information is transduced from a mechanical signal to a neurally conducted electrical impulse by the action of neuro-epithelial **cells** (hair **cells**) and SGN in the **inner ear**. All central fibers of SGN form synapses in the cochlear nucleus of the pontine brain stem. The auditory projections from. . . to the auditory brain stem and the auditory cortex. All auditory information is transduced by a limited number of hair **cells**, which are the sensory receptors of the **inner ear**, of which the so-called inner hair **cells**, numbering a comparative few, are critically important, since they form synapses with approximately 90 percent of the primary auditory neurons.. . . nucleus, the number of neural elements involved is measured in the hundreds of thousands. Thus, damage to a relatively few **cells** in the auditory periphery can lead to substantial hearing loss. Hence, many causes of sensorineural loss can be ascribed to lesions in the **inner ear**. This hearing loss can be progressive. In addition, the hearing becomes significantly less acute because of

changes in the anatomy. . .

SUMM The toxic effects of these drugs on auditory **cells** and spiral ganglion neurons are often the limiting factor for their therapeutic usefulness. For example, antibacterial aminoglycosides such as gentamicins,. . . infection has reached advanced stages, but at this point permanent damage may already have been done to the middle and **inner ear** structure. Clearly, ototoxicity is a dose-limiting side-effect ofantibiotic administration. For example, nearly 75% of patients given 2 grams of streptomycin. . .

SUMM Accordingly, there exists a need for means to prevent, reduce or treat the incidence and/or severity of **inner ear** disorders and hearing impairments involving **inner ear** tissue, particularly **inner ear** hair **cells**, and optionally, the associated auditory nerves. Of particular interest are those conditions arising as an unwanted side-effect of ototoxic therapeutic. . . a method that provides a safe, effective, and prolonged means for prophylactic or curative treatment of hearing impairments related to **inner ear** tissue damage, loss, or degeneration, particularly ototoxin-induced and particularly involving **inner ear** hair **cells**. The present invention provides compositions and methods to achieve these goals and others as well.

SUMM The present invention is based in part on the discovery disclosed herein that the **inner ear** hair **cells** produced FGF-2 in vivo, that utricular epithelial **cells** expressed FGF receptor in vitro, and that administration of certain **growth** factors can stimulatethe production of new inner hair **cells** by inducing **proliferationof** supporting **cells** which are the hair **cell** progenitors. Among 30 **growth** factors examined, FGF-2 was the most potent mitogen. IGF-1 was also effective. Accordingly, it is an object of the invention to provide a means of inducing, promoting, or enhancing the **growth**, **proliferation**, or **regeneration** of **inner ear** tissue, particularly **inner ear** epithelial hair **cells**, in vitro, ex vivo or in vitro. It is a further object of the invention to provide a method for treating a mammal to prevent, reduce, or treat the incidence of or severity of an **inner ear** hair **cell**-related hearing impairment or disorder (or balance impairment), particularly an ototoxin-induced or -inducible hearing impairment, by administering to a mammal in. . . treatment a prophylactically or therapeutically effective amount of FGF-2, IGF-1, their agonists, a functional fragment or derivative thereof, a chimeric **growth** factor comprising FGF-2 or IGF-1, a small molecule or antibody agonist thereof, or a combination of the foregoing. Optionally, a. . . a suitable interval(s) either prior to, subsequent to, or substantially concurrently with the administration of or exposure to hearing-impairment inducing **inner ear** tissue damage, preferably ototoxin-induced or -inducible hearing impairment.

DETD . . . refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) **inner ear** tissue-damage-related hearing disorder or impairment (or balance impairment), preferably ototoxin-induced or inducible, and involving **inner ear** hair **cells**. Those in need of treatment include those already experiencing a hearing impairment, those prone to having the impairment, and those in which the impairments are to be prevented. The hearing impairments are due to **inner ear** hair **cell**

damage or loss, wherein the damage or loss is caused by infections, mechanical injury, loud sounds, aging, or, preferably, chemical-induced. . .

DETD . . . in turn impairs hearing (and/or balance). In the context of the present invention, ototoxicity includes a deleterious effect on the **inner ear** hair **cells**. Ototoxic agents that cause hearing impairments include, but are not limited to, neoplastic agents such as vincristine, vinblastine, cisplatin, taxol,. . .

DETD The patients targeted for treatment by the current invention include those patients with **inner ear** hair **cell** related conditions as defined herein.

DETD Hearing impairments relevant to the invention are preferably sensory hearing loss due to end-organ lesions involving **inner ear** hair **cells**, e.g., acoustic trauma, viral endolymphatic labyrinthitis, Meniere's disease. Hearing impairments include tinnitus, which is a perception of sound in the. . . and adenoviruses. The hearing loss can be congenital, such as that caused by rubella, anoxia during birth, bleeding into the **inner ear** due to trauma during delivery, ototoxic drugs administered to the mother, erythroblastosis fetalis, and hereditary conditions including Waardenburg's syndrome and. . . syndrome. The hearing loss can be noise-induced, generally due to a noise greater than 85 decibels (db) that damages the **inner ear**. Hearing loss includes presbycusis, which is a sensorineural hearing loss occurring as a normal part of aging, fractures of the. . . rupturing the tympanic membrane and possibly the ossicularchain, fractures affectingthe cochlea, and acoustic neurinoma, which are tumors generally of Schwann **cell** origin that arise from either the auditory or vestibular divisions of the 8th nerve. Preferably, the hearing loss is caused by an ototoxic drug that effects the auditory portion of the **inner ear**, particularly **inner ear** hair **cells**. Incorporated herein by reference are Chapters 196, 197, 198 and 199 of The Merck Manual of Diagnosis and Therapy, 14th. . .

DETD Studies in lower vertebrates and avian systems indicate that supporting **cells** in the inner ears are hair **cell** progenitors (see for example, 27 and 49). In response to injury supporting **cells** are induced to **proliferate** and differentiate into new hair **cells**. However, in the mammalian system, supporting **cell proliferation** and hair **cell regenerating** occurs at a much lower frequency than in the avian system (48, 92, 127). The mammalian utricular epithelial supporting **cells** express epithelial antigens, including the tightjunction protein (ZO1), cytokeratin, and F-actin, but not fibroblast antigens, vimentin and Thy1.1 or glial **cell** and neuronal antigens. Characteristically,in culture, supporting **cells** require **cell**-to-**cell** contact for survival, which can be provided by other supporting **cells**, and by a fibroblast monolayer as observed with dissociated chick cochlear epithelial **cells** (16). Identification of the molecular and cellular mechanisms underlying the development and **regeneration** ofhair **cells**, has been hampered by the small tissue size, the complicated bony structures of the **inner ear**, and by the lack of hair **cell** progenitor culture systems.

DETD . . . a mammal prophylactically to prevent or reduce the occurrence or severity of a hearing (or balance) impairmentthat would result from **inner ear cell** injury, loss, or degeneration, preferably caused by an ototoxic agent, wherein a therapeutically effective amount of a **inner ear**

supporting **cell growth** factor or agonist of the invention, which are compounds that promote hair **cell regeneration, growth, proliferation**, or prevent or reduce cytotoxicity of hair **cells** by induction of the **proliferation** of supporting epithelial **cells** leading to **generation** of new hair **cells**. Such molecules are agonists of the utricular epithelial **cell** FGF- and IGF-1-high-affinity binding receptors that were identified herein as expressed on the surface of sensory epithelium **cells**. Preferred compounds are FGF-2, IGF-1, agonists thereof, a functional fragment or derivative thereof, a chimeric **growth** factor comprising FGF-2 or IGF-1, such as those containing the receptor-binding sequences from FGF-2 or IGF-1, a small molecule mimic. . . or a combination of the foregoing. Optionally, a trkB or trkC agonist is also administered to the mammal when neuronal **cell** damage is also suspectedor expected. Preferablythe trkB or trkC agonist is a neurotrophin, more preferably neurotrophin NT-4/5, NT-3, or BDNF,. . . at least 80% of the binding of the natural neurotrophin ligand to the receptor. When the patient is human, the **growth** factors and neurotrophins are preferably human **growth** factors and neurotrophins or derived from human gene sequences, in part to avoid or minimize recognition of the agonist as. . .

DETD Also provided herein are methods for promoting new **inner ear** hair **cells** by inducing **inner ear** supporting **cell proliferation regeneration** , or **growth** upon, prior to, or after exposure to an agent or effect that is capable of inducing a hearing or balance. . . impairment or disorder. Such agents and effects are those described herein. The method includes the step of administering to the **inner ear** hair **cell** an effective amount of FGF-2, IGF-1, or agonist thereof, or or factor disclosed herein as useful. Preferably, the method is. . .

DETD . . . of each component for purposes herein are thus determined by such considerations and are amounts that preventdamage or degeneration of **inner ear cell** function or restore **inner ear cell** function.

DETD . . . or infusions. As with the FGF-2, the IGF-I may be formulated so as to have a continual presence in the **inner ear** during the course of treatment, as described above for FGF-2. Thus, it may be covalently attached to a polymer, made into a sustained-release formulation, or provided by implanted **cells** producing the factor.

DETD Delivery oftherapeutic agents to the **inner ear** of a subject can be done by contact with the **inner ear** or through the external auditory canal and middle ear, as by injection or via catheters, or as exemplified in U.S. Pat. No. 5,476,446, which provides a multi-functional apparatus specifically designed for use in treating and/or diagnosing the **inner ear** of a human subject. The apparatus, which is useful in the practice of the present invention, has numerous functional capabilities including but not limited to (1) deliveringtherapeutic agents into the **inner ear** or to middle-**inner ear** interface tissues; (2) withdrawing fluid materials from the **inner ear**; (3) causing temperature, pressure and volumetric changes in the fluids/fluid chambers of the **inner ear**; and (4) enabling **inner ear** structures to be electrophysiologically monitored. In addition, other systems may be used to deliver the factros and formulations of the. . . Calif. (USA).

U.S. Pat. No. 4,892,538, provides an implantation device for delivery of the factors and formulations of the invention. **Cells** genetically engineered to express FGF-2, or IGF-1, or their combination, and optionally, enhancing or augmenting factors or therapeutics (e.g., trkB or trkC agonist), can be implanted in the host to provide effective levels of factor or factors. The **cells** can be prepared, encapsulated, and implanted as provided in U.S. Pat. Nos. 4,892,538, and 5,011,472, WO 92/19195, WO 95/05452, or. . .

DETD . . . a solution that is isotonic with the blood of the recipient, and even more preferably formulated for local administrationto the **inner ear**. Examples of carrier vehicles include water, saline, Ringer's solution, a buffered solution, and dextrose solution. Non-aqueous vehicles such as fixed. . . amounts of additives such as substances that enhance isotonicity and chemical stability, and when locally administered are non-toxic to the **cells** and structures of the **ear**, particularly the **inner ear**. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate,. . .

DETD . . . embodiment, agonist compositions of the invention are used during clinical organ implants or transplants to keep or improve viability of **inner ear** hair **cells**. Preferably a combination of a factors will be used as taught herein, including a trkB and a trkC agonist, with. . .

DETD . . . the Examples section herein, intact utricular epithelial sheets separated using a combined enzymatic and mechanical method essentially contain only supporting **cells** and hair **cells** (Corwin et al., 1995). The epithelial identity of the cultured **cells** was confirmed using various specific **cell** markers. While these **cells** expressed epithelial antigens including the tight junction protein (ZO1), cytokeratin and F-actin, they did not express fibroblast antigens, vimentin and Thy1.1, or glial and neuronal antigens. Most of the hair **cells** (stereocilliary bundle-bearing **cells**) were injured and many of them were dead after 2 days in culture due to their sensitivity to enzymatic digestion and mechanical trituration. Therefore, these cultures essentially represented a population of utricular supporting **cells** which are the progenitors for hair **cells** (Corwin and Cotanche, 1988; Balak et al., 1990; Rapheal, 1992; Weisleder and Rubel, 1992). These cultures provide an in vitro system to study **proliferation** and differentiation of the **inner ear** supporting **cells**.

DETD The cultured **inner ear** epithelial **cells** required **cell-cell** contacts with neighboring epithelial **cells** to survive and **proliferate**. Initial attempts to culture completely-dissociatedepithelial **cells** led to virtually all **cells** dying. A requirement ofcell-cellcontact for the survival and **proliferation** of epithelial progenitors is not unprecedented and has been observed previously with brain germinal zone progenitor **cells** (Gao et al., 1991) and E9 rat neuroepithelial **cells** (Li et al., 1996). The fact that **proliferation** of neuroepithelial **cells** only occurs within the highly compact CNS ventricular zone in vivo, and in the progenitor reaggregates (Gao et al., 1991) or neurospheres (Reynolds and Weiss, 1992) in vitro, suggests the existence of a membrane-bound factor for the **growth** of neuroepithelialcells. Consistent with this idea, membrane-bound components from a C6 glioma **cell** line have been shown to be necessary for the **proliferation** and survival of dissociated, single cortical progenitorcells (Davis and

Temple, 1994). In contrast to the organ culture (Warchol and Corwin, 1993). the partially dissociated epithelial **cells** grew poorly in serum-free medium, suggesting that in addition to the membrane bound molecules, soluble factors in the serum also promote the **growth** of these **cells**. A monolayerof fibroblast **cells** was reported as sufficient to support the **growth** of completely-dissociated chick cochlear epithelial **cells** (Finley and Corwin, 1995).

DETD The pure epithelial **cell** culture, along with the tritiated thymidine assay, was a rapid and convenient method to evaluateeffects of **growth** factors on **proliferation** of the **inner ear** epithelial progenitorcells. A large panel of agents could be and were examined in a relatively short time. The results of. . . data. In the present experiments, several FGF family members, namely IGF-1, IGF-2, TGF-.alpha. and EGF, were mitogenic factors for the **proliferation** of utricular supporting **cells**, from among 30 **growth** factors.

DETD . . . (1995) in the intact organ culture. One possibility for the discrepancy between these results is that the deprivation of hair **cells** in the present dissociated utricular epithelial **cell** cultures might trigger the upregulation of FGF and IGF-1 receptors and enhance the response to FGFs and IGF-1. If so, this likely reflects the situation occurring during **inner ear** injury or assault. Recently, Lee and Cotanche (1996) reported that damaging chicken cochlear epithelium by noise results in an upregulation of mRNA for the FGF receptor in the supporting **cells**. Finley and Corwin (1995) reported that FGF-2 promotes the **proliferation** of chick cochlear supporting **cells** which were completely dissociated and plated on a monolayer of fibroblast **cells**. The presence of high levels of FGF receptor and IGF-1 receptor in the **inner ear** epithelial **cells** after deprivation of hair **cells** and the inhibition of **cell proliferation** by neutralizing antibodiesaagainsteither FGF-2 or IGF-1 support the idea that FGF-2 and IGF-1 act directly on the **inner ear** supporting **cells** and induce their **proliferation** following the removal of hair **cells**. FGF-2 and IGF-1 may be candidate molecules regulating **proliferation** of the **inner ear** supporting **cells**, particularly during hair **cell regeneration** following challenge by aminoglycosides or noise.

DETD Alternatively, there may be a developmental response change to **growth** factors including FGF-2 and IGF-1 during maturation of the **inner ear** epithelium. It is possible that the mature **inner ear** epithelium responds differently relative to the developing epithelium. Exogenously added FGF-2 or IGF-1 might not elicit a **proliferation** in the intact, mature utricles (Yamashita and Oesterle, 1995) or in chick tissues which are treated with a very low. . . al., 1996) as they would in the immature utricles. Upon intensive damage by noise or drugs (massive degeneration of hair **cells**), the immature epithelium might be triggered to go back to an earlier developmental stage. Such injury induced status shift has been noticed for developing neurons (Gao and Macagno, 1988). The present study is performed on postnatal rat **inner ear cells** which are still undergoing maturation, but nonetheless is believed probativeto the influenceof FGF-2 and IGF-1 on hair **cell regeneration** after acoustic trauma or exposure to high doses of aminoglycosides in adult mammals.

DETD The finding that utricular epithelial **cells** express FGF-2 and

its receptor indicates that FGF-2 is a physiological **growth** factor for the development, maintenance and/or **regeneration** of hair **cells**. FGF-2 may exert its action through an autocrine mechanism. In this model, FGF-2 produced from hair **cells** may provide their own trophic support. Recent studies have suggested that **cell** differentiation and survival in the nervous system can be regulated by a **growth** factor-mediated autocrine interaction. For instance, colocalization of neurotrophins and their mRNAs is found in developing rat forebrain (Miranda et al., 1993) and a BDNF autocrine loop regulatesthe survival of cultured dorsal root ganglion **cells** (Acheson et al., 1995). Low et al. (1995) suggested that FGF-2 protects postnatal rat cochlear hair **cells** from aminoglycoside induced injury. Alternatively, a paracrine action might also be postulated in which FGF-2 synthesized by hair **cells** could locally influence maintenance of neighboring hair **cells** and **proliferation** of supporting **cells**. In this case, degeneration of hair **cells** may lead to a burst release of FGF-2, which would in turn stimulate supporting **cell** **proliferationin** the **inner ear** epithelium. The latter hypothesis may explaïn the supporting **cell** **proliferation** following hair **cell** death due to acoustic trauma or exposure to aminoglycosides, since FGF-2 does not have a signal sequence and **cell** injury is a major way for its release. The data herein that anti-FGF-2 antibody, but not anti-TGF-.alpha. antibody, significantly inhibits **cell** **proliferation** (FIG. 7) supports this hypothesis to a certain extent. The partial, but notcomplete, blocking effect by anti-FGF-2 antibody could be attributable to possible existence of other mitogens in the culture, loss of FGF-2 (due to hair **cell** injury) during the dissociation process and/or relief from contact inhibition within the epithelium following dissociation.

DETD Similar to neurotrophins, many other **growth** factors examined in the present experiments do not show significant mitogenic effects on utricular supporting **cells**. They could, however, still be involved in later phases of hair **cell** **regeneration**. For example, retinoic acid can induce formation of supernumerary hair **cells** in the developing cochlea without involvement of **cell** **proliferation** (Kelley et al., 1993). On the other hand, early differentiating factors might inhibit the progenitor **proliferation** and push the progenitors to come out the **cell** cycle and become postmitotic **cells**. Regarding this aspect, it is interesting to note then that TGF-.beta.1, TGF-.beta.2, TGF-.beta.3 and TGF-.beta.5 exhibit an inhibition on the **proliferation** of the **inner ear** epithelial **cells**. Whether such observation implies a possible involvement of TGF-.beta.s in the differentiation of hair **cells** remains to be determined.

DETD In summary, we have established a purified mammalian utricular epithelial **cell** culture, which allowed rapid examination of effects of **growth** factors on supporting **cell** **proliferation**, an early phase during normal development and **regeneration** of new hair **cells**. Among the 30 **growth** factors we examined, FGF-2 is the most potent mitogen. The observation that the **inner ear** hair **cells** produced FGF-2 in vivo and utricular epithelial **cells** expressed FGF receptor in vitro suggest a physiological role of FGF-2 in hair **cell** development, maintenance or **regeneration**.

DETD . . . recombinant neurotrophins (Genentech), TGF-.beta.1 (Genentech), TGF-.beta.2, TGF-.beta.3, TGF-.beta.5 (R & D Systems), activin, inhibin, glial cell derived neurotrophic factor (GDNF), **heregulin**, Gas-6, vascular endothelial growth factor (VEGF), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1, c-kit ligand (Genentech), platelet-derived growth. . .

DETD . . . growth factors have been reported to influence cell proliferationand differentiation. These include neurotrophins,the TGF-.beta. superfamily, glial cell mitogens such as **heregulin** and Gas-6, endothelial cell mitogen such as VEGF, and others listed in Table 3. When examined in these cultures, none. . .

DETD . . . 126

TGF-.beta.5 807 .+-. 59
Activin 2383 .+-. 186
Inhibin 1959 .+-. 183
GDNF 2383 .+-. 186
Schwann cell mitogens
Heregulin 2854 .+-. 179
Gas-6 2588 .+-. 95
Endothelial cell mitogen
VEGF 2156 .+-. 211
PDGF 2387 .+-. 299
CNTF 2918. . .

CLM What is claimed is:

1. A method for increasing the number of mammalian **inner ear** hair **cells**, comprising contacting mammalian **inner ear** supporting **cells** with an amount of FGF-2 that promotes **proliferation** of said **inner ear** supporting **cells**.

2. The method of claim 1 further comprising contacting said **inner ear** supporting **cells** with a supporting **cell proliferation**-inducing amount of TGF-.alpha. or a TGF-.alpha.-receptor agonist.

5. The method of claim 1, further comprising contacting said **inner ear** supporting **cells** with IGF-1 or an IGF-1 receptor agonist.

10. A method for treating a mammalian **inner ear** hair **cell** related disorder in a mammal comprising administering to the mammal an effective amount of FGF-2 that promotes **proliferation** of **inner ear** supporting **cells**.

L3 ANSWER 5 OF 6 USPATFULL

AB A non-naturally occurring peptide derived from EGF-like domains of NDF/ **heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. A monoclonal antibody against adult rat utricular epithelium is also described.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

ACCESSION NUMBER: 2000:80853 USPATFULL
TITLE: Monoclonal antibody against utricular epithelium
INVENTOR(S): Carnahan, Josette F., Newbury Park, CA, United States
PATENT ASSIGNEE(S): Amgen Inc., Thousands Oaks, CA, United States (U.S.

corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6080845		20000627
APPLICATION INFO.:	US 1999-238182		19990128 (9)

RELATED APPLN. INFO.: Continuation-in-part of Ser. No. US 1998-129549, filed on 5 Aug 1998, now abandoned
DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Huff, Sheela
LEGAL REPRESENTATIVE: Mazza, Richard J., Levy, Ron K., Odre, Steven M.
NUMBER OF CLAIMS: 1
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 6 Drawing Figure(s); 5 Drawing Page(s)
LINE COUNT: 672
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. A monoclonal antibody against adult rat utricular epithelium is also described.

SUMM This invention relates to the NDF/**heregulin** protein family, and more specifically to the use of a derivative peptide to stimulate the **proliferation** of sensory epithelial **cells** of the **inner ear** for the treatment of vestibular disorders. The invention also relates to monoclonal antibodies raised against adult rat utricular epithelium.

SUMM . . . Letters, Volume 349, pages 139-143 (1994); and Carraway et al., Journal of Biological Chemistry, Volume 269, pages 14303-14306 (1994). The NDF/**heregulin** family is considered to also include ARIA and glial growth factor (GGF); see, respectively, Falls et al., Cell, Volume 72,. . .

SUMM The present invention comprises the use of a peptide of following sequence as a **growth** stimulant for sensory epithelial **cells** of the **inner ear**:

SUMM . . . a hybrid form derived from the EGF-like domains of NDF-.alpha. and NDF-.beta.. However, the usefulness of this molecule as a **growth** stimulant for sensory epithelial **cells** of the utricle in the **inner ear**, which is demonstrated in the working examples below, has not been previously recognized. Because all of the vestibular organs (e.g.,. . . peptide may also be useful to treat hearing loss in mammals, including humans, which is attributable to the degeneration of **inner ear** hair **cells**, i.e., by **regenerating** such hair **cells** in association with sensory epithelium.

DRWD . . . a graph comparing the mitogenic activity (as BrdU-positive nuclei) of the peptide of SEQ ID NO: 1 ("Peptide") with other NDF/**heregulin**-derived peptides on **inner ear** sensory epithelial **cells**.

DETD The mitogenic activity of the peptide of SEQ ID NO: 1 on the vestibular sensory epithelium of the mammalian **inner ear** suggests that it may also be useful to **regenerate** hair **cells**, which are critical for hearing. Thus, the peptide may be beneficial for treating hearing loss associated with deteriorated or damaged **inner ear** hair **cells**, and such applications are included within the therapeutic treatments made possible by the present invention.

DETD Sensory epithelial **cells** obtained from utricles in the **inner ear** of both seven day-old (infant) rats and six week-old (adult) rats were isolated with the use of thermolysin treatment; see. . . page 87 (1995). All edges were trimmed away and the central portion of the epithelium was cut into quarters. Epithelial **cells** from the infant rats were cultured in DMEM/F12 with 10% FBS (Gibco BRL, Grand Island, N.Y.), and 3 micrograms per. . . 1 or 50 ng/ml of recombinant derived FGF-10, recombinant derived FGF-16, recombinant derived ciliary-derived neurotrophic factor (CNTF), recombinant derived neurotrophic **growth** factor (NGF), recombinant derived glial-derived neurotrophic factor (GDNF), recombinant derived keratinocyte **growth** factor (KGF), or a control (no **growth** factor present). The experiment was ended by fixing in 4% paraformaldehyde for one hour.

DETD Using the test procedure of Example 1, the peptide of SEQ ID NO: 1 was compared with members of the NDF-**heregulin** family in primary cultures of young rat utricular sensory epithelial cells, at a treatment concentration of 50 ng/ml in each. . .

DETD **Generation** of Monoclonal Antibodies Against Sensory Epithelial **Cells** of Rodent **Inner Ear**

DETD The lack of a specific marker for sensory epithelium **cells** adds to the challenges associated with research on hair **cell regeneration** in the **inner ear** of mammals. Monoclonal antibodies against hair **cells** have been reported in the literature; Finley et al., Assoc. Res. Otolaryngol. Abstr., Volume 20, page 16 (1997) and Holley et al., J. Neurocytol., Volume 24, pages 853-864 (1997). However, none of these antibodies are specific to supporting **cells** in the mammalian vestibular organs.

DETD . . . is a description of the preparation of four distinct monoclonal antibodies raised against rat utricular epithelia which specifically label supporting **cells** of the vestibular organs in the **inner ear** of the rodent. These antibodies constitute an additional aspect of the present invention.

DETD In this method, sensory epithelia were isolated from adult rat **inner ear** utricles by the thermolysin method; see above for description. Seventy pieces of epithelia were homogenized by ultrasound, and then emulsified. . . showed high titer against the antigen (i.e., the utricle extract). Mouse splenocytes were harvested and then fused with HL-1 myeloma **cells** (Kohler and Milstein, Nature, Volume 256, pages 495-497 (1975). Screening for monoclonal antibodies was conducted by immunostaining on frozen 10-micron. . .

DETD Each of the monoclonal antibodies specifically stained the supporting **cells**, but with a characteristically different pattern. SC-1 stained the top portion of the supporting **cells** brightly, while gradually decreasing around the **cell** nuclei. SC-2 stained only the top portion of the supporting **cells**. SC-3 immunoreactivity was concentrated on the lower cytoplasmic portion of the supporting **cells** in neotal rat utricles, and migrated to the upper portion in adult utricles. SC-4 immunoreactivity was found mostly in the supporting **cell** apex of the adult utricle. SC-4 and SC-3 immunostaining was found in embryonic progenitors of supporting **cells** of the **inner ear**.

L3 ANSWER 6 OF 6 CAPLUS COPYRIGHT 2002 ACS

AB Hair **cell** loss due to acoustic and ototoxic damage often leads to hearing and balance impairments. Although a spontaneous even in chicks and lower vertebrates, hair **cell** replacement occurs at a much lower frequency in mammals presumably due to a very low rate of supporting

cell proliferation following injury. The authors report that **heregulin**, a member of the neuregulin family, dramatically enhances **proliferation** of supporting **cells** in postnatal rat utricular epithelial sheet cultures after gentamicin treatment, as revealed by bromo-deoxyuridine (BrdU) immunocytochem. A dose-dependent study shows that the maximal effects of **heregulin** are achieved at 3 nM. The mitogenic effects of **heregulin** are confirmed in utricular whole mount cultures. Autoradiog. of the utricular whole mount cultures shows that **heregulin** also enhances the no. of tritiated thymidine-labeled **cells** within the hair **cell** layer. TaqMan quant. RT-PCR anal. and immunocytochem. reveal that **heregulin** and its binding receptors (ErbB-2, ErbB-3 and ErbB-4) are expressed in the **inner ear** sensory epithelium. Of several ligands activating various ErbB receptors, including **heregulin**, neuregulin-3, .beta.-cellulin, heparin binding-epidermal **growth** factor (HB-EGF), transforming **growth** factor-.alpha. (TGF-.alpha.) and EGF, **heregulin** shows the most potent mitogenic effects on supporting **cells**. Because neuregulin-3 that signals only through ErbB-4 does not show an effect, these data suggest that activation of the ErbB-2-ErbB-3 heterodimeric complexes, rather than ErbB-4, is crit. for the **proliferative** response in the utricular sensory epithelium. In addn., gentamicin treatment induces an upregulation of **heregulin** mRNA. Considered together, **heregulin** may play an important role in hair **cell regeneration** following ototoxic damage.

ACCESSION NUMBER: 2000:652691 CAPLUS
DOCUMENT NUMBER: 133:345081
TITLE: **Heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage
AUTHOR(S): Zheng, J. Lisa; Frantz, Gretchen; Lewis, Annette K.; Sliwkowski, Mark; Gao, Wei-Qiang
CORPORATE SOURCE: Department of Neuroscience, Genentech Inc., South San Francisco, CA, 94080, USA
SOURCE: Journal of Neurocytology (2000), Volume Date 1999, 28(10/11), 901-912
CODEN: JNCYA2; ISSN: 0300-4864
PUBLISHER: Kluwer Academic Publishers
DOCUMENT TYPE: Journal
LANGUAGE: English
REFERENCE COUNT: 58 THERE ARE 58 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI **Heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage

AB Hair **cell** loss due to acoustic and ototoxic damage often leads to hearing and balance impairments. Although a spontaneous even in chicks and lower vertebrates, hair **cell** replacement occurs at a much lower frequency in mammals presumably due to a very low rate of supporting **cell proliferation** following injury. The authors report that **heregulin**, a member of the neuregulin family, dramatically enhances **proliferation** of supporting **cells** in postnatal rat utricular epithelial sheet cultures after gentamicin treatment, as revealed by bromo-deoxyuridine (BrdU) immunocytochem. A dose-dependent study shows that the maximal effects of **heregulin** are achieved at 3 nM. The mitogenic effects of **heregulin** are confirmed in utricular whole mount cultures. Autoradiog. of the utricular whole mount cultures shows that **heregulin** also enhances the no. of tritiated thymidine-labeled **cells** within the hair

cell layer. TaqMan quant. RT-PCR anal. and immunocytochem. reveal that **heregulin** and its binding receptors (ErbB-2, ErbB-3 and ErbB-4) are expressed in the **inner ear** sensory epithelium. Of several ligands activating various ErbB receptors, including **heregulin**, neuregulin-3, .beta.-cellulin, heparin binding-epidermal **growth** factor (HB-EGF), transforming **growth** factor-.alpha. (TGF-.alpha.) and EGF, **heregulin** shows the most potent mitogenic effects on supporting **cells**. Because neuregulin-3 that signals only through ErbB-4 does not show an effect, these data suggest that activation of the ErbB-2-ErbB-3 heterodimeric complexes, rather than ErbB-4, is crit. for the **proliferative** response in the utricular sensory epithelium. In addn., gentamicin treatment induces an upregulation of **heregulin** mRNA. Considered together, **heregulin** may play an important role in hair **cell regeneration** following ototoxic damage.

ST **heregulin** utricular sensory epithelium regeneration proliferation ototoxic damage; hair cell proliferation regeneration ototoxic damage

IT Cell proliferation
Regeneration, animal
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-3, heterodimeric complexes with ErbB-2; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BOC (Biological occurrence); BSU (Biological study, unclassified); BIOL (Biological study); OCCU (Occurrence)
(**heregulin**, ErbB-4; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-4; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BOC (Biological occurrence); BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); OCCU (Occurrence); PROC (Process)
(**heregulin**, erbB-3, heterodimeric complexes with ErbB-2; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT neu (receptor)
RL: BOC (Biological occurrence); BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); OCCU (Occurrence); PROC (Process)
(heterodimeric complexes with ErbB-3; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT **Ear**

(**inner**, sensory epithelium; **heregulin** enhances **regenerative proliferation** in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Hearing
(loss; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(neuregulin-3; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Ear
(organ of Corti, hair cell; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Ear
(ototoxicity; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Ear
(utriculus; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Transforming growth factors
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(.alpha.-; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT 1403-66-3, Gentamicin
RL: ADV (Adverse effect, including toxicity); BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after gentamicin-induced ototoxic damage)

IT 62229-50-9, Epidermal growth factor 154531-34-7, Heparin-binding epidermal growth factor-like growth factor 163150-12-7, BetaCellulin
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

FILE 'CAPLUS' ENTERED AT 11:13:13 ON 05 DEC 2002
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FILE 'USPATFULL' ENTERED AT 11:13:13 ON 05 DEC 2002

=> s l3 and (HER2 or HER3)
L4 3 L3 AND (HER2 OR HER3)

=> d l4 abs ibib kwic 1-3

L4 ANSWER 1 OF 3 CAPLUS COPYRIGHT 2002 ACS
AB Ligands which bind to the **HER2** and/or **HER3** receptors are useful as **inner-ear**-supporting **cell-growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**, e.g. in treatment of hearing disorders. Thus, in cultures of rat utricular epithelial sheets, **heregulin** HRG-.beta.1-177-244 significantly enhanced **proliferation** of utricular supporting **cells**. In chinchillas, **heregulin** acts in vivo to enhance **inner ear** supporting **cell proliferation** and hair **cell generation** following ototoxic injury and acoustic assault. Heregulins may also be used ex vivo for expansion of supporting **cells**, followed by reimplantation into the **inner ear**.

ACCESSION NUMBER: 2000:335267 CAPLUS
DOCUMENT NUMBER: 133:814
TITLE: Method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for **HER2** and/or **HER3** receptors
INVENTOR(S): Gao, Wei-qiang
PATENT ASSIGNEE(S): Genentech, Inc., USA
SOURCE: PCT Int. Appl., 141 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000027426	A1	20000518	WO 1999-US25744	19991028
W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
CA 2345899	AA	20000518	CA 1999-2345899	19991028
EP 1126873	A1	20010829	EP 1999-956853	19991028
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,				

IE, SI, LT, LV, FI, RO
US 2002081299 A1 20020627 US 2001-849868 20010504
PRIORITY APPLN. INFO.: US 1998-107522P P 19981107
WO 1999-US25744 W 19991028
REFERENCE COUNT: 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI Method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for **HER2** and/or **HER3** receptors

AB Ligands which bind to the **HER2** and/or **HER3** receptors are useful as **inner-ear**-supporting **cell-growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**, e.g. in treatment of hearing disorders. Thus, in cultures of rat utricular epithelial sheets, **heregulin** HRG-.beta.1-177-244 significantly enhanced **proliferation** of utricular supporting **cells**. In chinchillas, **heregulin** acts in vivo to enhance **inner ear** supporting **cell proliferation** and hair **cell generation** following ototoxic injury and acoustic assault. Heregulins may also be used ex vivo for expansion of supporting **cells**, followed by reimplantation into the **inner ear**.

ST ear hair **cell regeneration heregulin**; HER receptor ligand **inner ear**; **cell proliferation inner ear heregulin**

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.alpha.; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.1; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.2-like; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.2; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(HRG-.beta.3; enhancing **proliferation** of **inner**

ear hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Ear
(cochlea, implant; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT **Ear**
(disease; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Animal tissue culture
Molecular cloning
(enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT **Growth** factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-3, ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT **Growth** factor receptors
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(**heregulin**, erbB-3, ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Antibodies
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**heregulin**-agonistic; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT Drug delivery systems
(implants, cochlear; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT **Ear**
(**inner**, supporting **cell**; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT **Ear**
(**inner**, utricle; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)

IT neu (receptor)
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or

HER3 receptors)
IT Ear
(organ of Corti, hair **cell**; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)
IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(.gamma.-HRG; enhancing **proliferation** of **inner ear** hair **cells** with ligands for **HER2** and/or **HER3** receptors)
IT 142158-51-8 142158-52-9 142158-53-0 146591-70-0 146591-82-4 178862-39-0 196678-45-2 270245-15-3 270245-16-4, 14: PN: WO0027426 SEQID: 2 unclaimed DNA 270245-18-6
RL: PRP (Properties)
(unclaimed nucleotide sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for **HER2** and/or **HER3** receptors)
IT 146591-75-5, **Heregulin** .beta.2 (human clone .lambda.her76 precursor reduced) 146591-80-2, Protein (human clone .lambda.her84 **heregulin** .beta.2-like precursor reduced) 168183-94-6 198086-50-9, **Heregulin** (human gene .gamma.-HRG) 270245-14-2 270245-17-5
RL: PRP (Properties)
(unclaimed protein sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for **HER2** and/or **HER3** receptors)
IT 146591-69-7, 1-625-**Heregulin** .alpha. (human clone .lambda.gt10her16 precursor reduced) 146591-71-1 146591-78-8, **Heregulin** .beta.3 (human clone .lambda.her78 precursor reduced) 260348-98-9 270560-38-8 270560-39-9 270560-40-2
RL: PRP (Properties)
(unclaimed sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for **HER2** and/or **HER3** receptors)

L4 ANSWER 2 OF 3 USPATFULL
AB Ligands which bind to the **HER2** and/or **HER3** receptors are useful as **inner-ear**-supporting **cell growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.
ACCESSION NUMBER: 2002:156704 USPATFULL
TITLE: Hair cell disorders
INVENTOR(S): Gao, Wei-Qiang, Foster City, CA, UNITED STATES

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 2002081299	A1	20020627	
APPLICATION INFO.:	US 2001-849868	A1	20010504	(9)

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PRIORITY INFORMATION:	US 1998-107522P	19981107 (60)

DOCUMENT TYPE: Utility

09/849,868

FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: KNOBBE MARTENS OLSON & BEAR LLP, 620 NEWPORT CENTER DRIVE, SIXTEENTH FLOOR, NEWPORT BEACH, CA, 92660
NUMBER OF CLAIMS: 18
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 34 Drawing Page(s)
LINE COUNT: 5225
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Ligands which bind to the **HER2** and/or **HER3** receptors are useful as **inner-ear**-supporting **cell growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**.

SUMM [0002] This application relates to inducing, promoting, or enhancing the **growth**, **proliferation**, repair, **generation**, or **regeneration** of **inner ear** tissue, particularly **inner ear** epithelial hair **cells** and supporting **cells**. More particularly, this application relates to potently stimulating supporting **cell proliferation** and enhancing **proliferation**-mediated **generation** of new hair **cells**. In addition, this application provides methods, compositions and devices for prophylactic and therapeutic treatment of **inner ear** disorders and conditions, particularly sensorineural hearing and balance impairments. This invention relates to the use of **HER2** ligands, in particular **heregulin** polypeptides, as **inner-ear**-supporting **cell growth** factors.

SUMM . . . a wide variety of causes, including infections, mechanical injury, loud sounds, aging, and chemical-induced ototoxicity that damages neurons and/or hair **cells** of the peripheral auditory system. The peripheral auditory system consists of auditory receptors, hair **cells** in the organ of Corti, and primary auditory neurons, the spiral ganglion neurons in the cochlea. Spiral ganglion neurons ("SGN") are primary afferent auditory neurons that deliver signals from the peripheral auditory receptors, the hair **cells** in the organ of Corti, to the brain through the cochlear nerve. The eighth nerve connects the primary auditory neurons. . . . are primary afferent sensory neurons responsible for balance and which deliver signals from the utricle, saccule and ampullae of the **inner ear** to the brain, to the brainstem. Destruction of primary afferent neurons in the spiral ganglia and hair **cells** has been attributed as a major cause of hearing impairments. Damage to the peripheral auditory system is responsible for a. . .

SUMM . . . nervous system may result in hearing loss or balance impairment. Auditory apparatus can be divided into the external and middle **ear**, **inner ear** and auditory nerve and central auditory pathways. While having some variations from species to species, the general characterization is common. . . . for all mammals. Auditory stimuli are mechanically transmitted through the external auditory canal, tympanic membrane, and ossicular chain to the **inner ear**. The middle **ear** and mastoid process are normally filled with air. Disorders of the external and middle ear usually produce a conductive hearing. . . . space. Auditory information is transduced from a mechanical signal to a neurally conducted electrical impulse by the action of neuro-epithelial **cells** (hair **cells**) and SGN in the **inner ear**. All central fibers of SGN form synapses in the cochlear nucleus of the pontine brain stem. The auditory projections from. . . to the auditory brain stem and the auditory cortex. All auditory

information is transduced by a limited number of hair **cells**, which are the sensory receptors of the **inner ear**, of which the so-called inner hair **cells**, numbering a comparative few, are critically important, since they form synapses with approximately 90 percent of the primary auditory neurons.. . . nucleus, the number of neural elements involved is measured in the hundreds of thousands. Thus, damage to a relatively few **cells** in the auditory periphery can lead to substantial hearing loss or balance impairment. Hence, many causes of sensorineural loss can be ascribed to lesions in the **inner ear**. This hearing loss and balance impairment can be progressive. In addition, the hearing becomes significantly less acute because of changes. . .

SUMM [0007] The toxic effects of these drugs on auditory **cells** and spiral ganglion neurons are often the limiting factor for their therapeutic usefulness. For example, antibacterial aminoglycosides such as gentamicins,. . . infection has reached advanced stages, but at this point permanent damage may already have been done to the middle and **inner ear** structure. Clearly, ototoxicity is a dose-limiting side-effect of antibiotic administration. For example, nearly 75% of patients given 2 grams of. . .

SUMM [0009] Accordingly, there exists a need for means to prevent, reduce or treat the incidence and/or severity of **inner ear** disorders and hearing impairments involving **inner ear** tissue, particularly **inner ear** hair **cells**, and optionally, the associated auditory nerves. Of particular interest are those conditions arising as an unwanted side-effect of ototoxic therapeutic. . . a method that provides a safe, effective, and prolonged means for prophylactic or curative treatment of hearing impairments related to **inner ear** tissue damage, loss, or degeneration, particularly ototoxin-induced, and particularly involving **inner ear** hair **cells**. The present invention provides compositions and methods to achieve these goals and others as well.

SUMM [0011] In general an object of the invention is to provide a method of inducing, promoting, or enhancing the **growth**, **proliferation**, repair, or **regeneration** of **inner ear** tissue, particularly **inner ear** hair **cells** and their supporting **cells** for the purpose of promoting repair and healing of inner tissue damage or injury.

SUMM [0012] Accordingly, one object of this invention is to provide a method of treating **inner ear** disorders and conditions in patients, primarily human patients, in need of such treatment. A further object is to provide a method of inducing **inner-ear** -supporting **cell growth**, **generation**, and development, which leads to **generation** of new hair **cells**.

SUMM . . . this invention, it has now been discovered that these objects and the broader objective of treating conditions associated with hair **cell** or **inner-ear**-supporting **cell** damage and injury are achieved by administering to a patient in need of such treatment an effective amount of a **heregulin** ligand, preferably a polypeptide or fragment thereof. These **heregulin** polypeptides, include HRG-.alpha., HRG-.beta.1, HRG-.beta.2, HRG-.beta.3 and other **heregulin** polypeptides which cross-react with antibodies directed against these family members and/or which are substantially homologous as defined below and includes **heregulin** variants such as N-terminal and C-terminal fragments thereof. A

preferred **heregulin** is the ligand disclosed in FIG. 1A-1D and further designated HRG-.alpha.. Other preferred heregulins are the ligands disclosed in FIG.. . .

SUMM [0014] In another aspect, the invention provides a method in which **heregulin** agonist antibodies are administered to achieve the objects of the invention. In this embodiment, **HER2/HER3** or fragments thereof (which also may be synthesized by in vitro methods) are fused (by recombinant expression or an in. . . vitro peptidyl bond) to an immunogenic polypeptide and this fusion polypeptide, in turn, is used to raise antibodies against a **HER2/HER3** epitope. Agonist antibodies are recovered from the serum of immunized animals. Alternatively, monoclonal antibodies are prepared from in vito cells. . . receptor, but will not substantially cross-react with any other known ligands such as EGF, and will activate the HER receptors **HER2** or **HER3**, preferably **Her2**. In addition, antibodies may be selected that are capable of binding specifically to individual family members of **heregulin** family, e.g. HRG-.alpha., HRG-.beta.1, HRG-.beta.2, HRG-.beta.3, and which are agonists thereof.

SUMM [0015] In general, the invention is a method of **regenerating** and/or repairing hair **cell** or **inner-ear**-supporting **cell** injury by stimulating **growth** and **proliferation** of **inner-ear**-supporting **cells** to enhance **generation** of new hair **cells**. The hair **cells** may be injured by many types of insults, for example, injury due to surgical incision or resection, chemical or smoke inhalation or aspiration, chemical or biochemical ulceration, **cell** damage due to viral or bacterial infection, etc The **inner-ear**-supporting **cells** which may be affected by the method of the invention include any **inner-ear**-supporting **cell** which expresses **HER2** or **HER3**, preferably **Her3**. The method of the invention stimulates **growth** and **proliferation** of the **inner-ear**-supporting **cells** leading to **generation** of new hair **cells** to repair and re-establish the sensorineural contacts in the **inner ear** to allow the affected tissues to develop normal physiological functions more quickly.

SUMM [0016] Accordingly, one embodiment of the invention is a method of inducing **inner-ear**-supporting **cell growth** by contacting a **inner-ear**-supporting **cell** which expresses **HER2** receptor with an effective amount of a **HER2** activating ligand.

SUMM [0017] A further embodiment is a method of treating **inner ear** hair **cell** injury, caused by ototoxins or acoustic assault for example, by administering to a patient in need thereof an effective amount of a **HER2** activating ligand.

DRWD . . . .beta.2-like and .beta.3 in descending order and illustrates the amino acid insertions, deletions, and substitutions that characterize these forms of **heregulin** (SEQ ID NOS: 1, 3, 5, 9, and 7).

DRWD [0027] FIG. 10 shows the dose-dependent proliferation effect of **heregulin** on cells in the rat utricular sheet hair cell layer, as indicated by the number of BrdU positive cells per. . .

DRWD . . . 11A-D show autoradiography of tritiated-thymidine labeled cells in supporting cell and hair cell layer in gentamicin-treated utricles in response to **heregulin** treatment. FIGS. A-D are views from similarly treated organotypic rat utricular whole mounts.

DRWD . . . tritiated-thymidine labeled cells in supporting cell and hair cell layer in gentamicin-treated utricles (shown in FIGS. 11A-D) in response to **heregulin** treatment compared to control.

DRWD [0030] FIG. 13 shows the RNA concentration of **heregulin** and the recepters **Her2**, **Her3** and Her4 in RAN isolated from the inner ear sensory epithelium layer.

DRWD [0031] FIG. 14 shows localization of **Her2**, a **heregulin** receptor, in the inner ear sensory epithelium, as indicated by immunostaining the P0 cochlea and adult utricle with labeled monoclonal antibody to **Her2**.

DETD [0032] **Heregulin** ligands, in particular polypeptides and agonist antibodies thereof, have affinity for and stimulate the **HER2**, and less preferably **HER3**, receptors or combinations thereof in autophosphorylation. Included within the definition of **heregulin** ligands, in addition to HRG-.alpha., HRG-.beta.1, HRG-.beta.2, HRG-.beta.3 and HRG-.beta.2-like, are other polypeptides binding to the **HER2** receptor, which bear substantial amino acid sequence homology to HRG-.alpha. or HRG-.beta.1. Such additional polypeptides fall within the definition of **heregulin** as a family of polypeptide ligands that bind to the **HER2** receptors.

DETD [0033] **Heregulin** polypeptides bind with varying affinities to the **HER2** receptors. It is also known that heterodimerization of **HER2** with **HER3** occurs with subsequent receptor cross-phosphorylation as described above. In the present invention, **inner-ear**-supporting **cell growth** and/or **proliferation** is induced when a **heregulin** protein interacts and binds with an individual receptor molecule or a receptor dimer such that receptor phosphorylation is induced. Binding and activation of **HER2** or **Her3** or combinations thereof, therefore, is meant to include activation of any form of the receptor necessary for receptor activation and. . . biologic function including monomeric receptor and dimeric receptor forms. Dimeric receptor forms may be referred to below, for example, as **HER2**/**HER3**.

DETD [0034] The HER (ErbB) family belongs to the subclass I receptor tyrosine kinase superfamily and consists of three distinct receptors, **HER2**, **HER3**, and HER4. A ligand for this ErbB family is the protein **heregulin** (HRG), a multidomain containing protein with at least 15 distinct isoforms.

DETD . . . as the product of the transforming gene from neuroblastomas of chemically treated rats. The neu gene (also called erbB2 and **HER2**) encodes a 185 kDa receptor protein tyrosine kinase. Amplification and/or overexpression of the human **HER2** gene correlates with a poor prognosis in breast and ovarian cancers (Slamon et al., Science 235:177-182 (1987); and Slamon et al., Science 244:707-712 (1989)). Overexpression of **HER2** has been correlated with other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon and bladder. Accordingly, Slamon et al. in U.S. Pat. No. 4,968,603 describe and claim various diagnostic assays for determining **HER2** gene amplification or expression in tumor cells. Slamon et al. discovered that the presence of multiple gene copies of **HER2** oncogene in tumor cells indicates that the disease is more likely to spread beyond the primary tumor site, and that. . . therefore require more aggressive treatment than might otherwise be indicated by other diagnostic factors. Slamon et al. conclude that the **HER2** gene amplification test, together with the determination of lymph node

status, provides greatly improved prognostic utility.

DETD [0037] A further related gene, called erbB3 or **HER3**, has also been described. See U.S. Pat. No. 5,183,884; Kraus et al., Proc. Natl. Acad Sci. USA 86:9193-9197 (1989); EP. . .

DETD [0039] The quest for the activator of the **HER2** oncogene has lead to the discovery of a family of **heregulin** polypeptides. These proteins appear to result from alternate splicing of a single gene which was mapped to the short arm. . .

DETD [0040] Holmes et al. isolated and cioned a family of polypeptide activators for the **HER2** receptor which they called **heregulin**-.alpha. (HRG-.alpha.), **heregulin**-.beta.1 (HRG-.beta.1), **heregulin**-.beta.2 (HRG-.beta.2), **heregulin**-.beta.2-like (HRG-.beta.2-like), and **heregulin** -.beta.3 (HRG-.beta.3). See Holmes et al., Science 256:1205-1210 (1992); WO 92/20798; and U.S. Pat. No. 5,367,060. The 45 kDa polypeptide, HRG-.alpha.,. . . from the conditioned medium of the MDA-MB-231 human breast cancer cell line. These researchers demonstrated the ability of the purified **heregulin** polypeptides to activate tyrosine phosphorylation of the **HER2** receptor in MCF7 breast tumor cells. Furthermore, the mitogenic activity of the **heregulin** polypeptides on SK-BR-3 cells (which express high levels of the **HER2** receptor) was illustrated. Like other growth factors which belong to the EGF family, soluble HRG polypeptides appear to be derived. . .

DETD [0042] Falls et al., Cell, 72:801-815 (1993) describe another member of the **heregulin** family which they call acetylcholine receptor inducing activity (ARIA) polypeptide. The chicken-derived ARIA polypeptide stimulates synthesis of muscle acetylcholine receptors. See also WO 94/08007. ARIA is a .beta.-type **heregulin** and lacks the entire spacer region rich in glycosylation sites between the Ig-like domain and EGF-like domain of HRG.alpha., and. . .

DETD . . . proteins which they call glial growth factors (GGFs). These GGFs share the Ig-like domain and EGF-like domain with the other **heregulin** proteins described above, but also have an amino-terminal kringle domain. GGFs generally do not have the complete glycosylated spacer region. . .

DETD [0044] Ho et al. in J. Biol. Chem. 270(4):14523-14532 (1995) describe another member of the **heregulin** family called sensory and motor neuron-derived factor (SMDF). This protein has an EGF-like domain characteristic of all other **heregulin** polypeptides but a distinct N-terminal domain. The major structural difference between SMDF and the other **heregulin** polypeptides is the lack in SMDF of the Ig-like domain and the "glyco" spacer characteristic of all the other **heregulin** polypeptides. Another feature of SMDF is the presence of two stretches of hydrophobic amino acids near the N-terminus.

DETD [0045] While the **heregulin** polypeptides were first identified based on their ability to activate the **HER2** receptor (see Holmes et al., supra), it was discovered that certain ovarian cells expressing neu and neu-transfected fibroblasts did not. . . undergo tyrosine phosphorylation (Peles et al., EMBO J. 12:961-971 (1993)). This indicated another cellular component was necessary for conferring full **heregulin** responsiveness. Carraway et al. subsequently demonstrated that .sup.125I-rHRG.beta.1.sub.177-244 bound to NIH-3T3 fibroblasts stably transfected with bovine erbB3 but not to. . . et al., J. Biol. Chem. 269(19):14303-14306 (1994). Sliwkowski et al., J. Biol. Chem. 269(20):14661-14665 (1994) found that cells transfected with **HER3** alone show low affinities for **heregulin**, whereas

cells transfected with both **HER2** and **HER3** show higher affinities.

DETD . . . researchers found that binding of EGF to the EGFR resulted in activation of the EGFR kinase domain and cross-phosphorylation of p185.sup.**HER2**. This is believed to be a result of ligand-induced receptor heterodimerization and the concomitant cross-phosphorylation of the receptors within the. . .

DETD [0047] Plowman and his colleagues have similarly studied p185.sup.HER4/p185.sup.**HER2** activation. They expressed p185.sup.**HER2** alone, p185.sup.HER4 alone, or the two receptors together in human T lymphocytes and demonstrated that **heregulin** is capable of stimulating tyrosine phosphorylation of p185.sup.HER4, but could only stimulate p185.sup.**HER2** phosphorylation in cells expressing both receptors. Plowman et al., Nature 336:473-475 (1993).

DETD [0048] The biological role of **heregulin** has been investigated by several groups. For example, Falls et al., (discussed above) found that ARIA plays a role in. . .

DETD . . . factor for astrocytes (Pinkas-Kramarski et al., PNAS, USA 91:9387-9391 (1994)). Meyer and Birchmeier, PNAS, USA 91:1064-1068 (1994) analyzed expression of **heregulin** during mouse embryogenesis and in the perinatal animal using in situ hybridization and RNase protection experiments. See also Meyer et al., Development 124(18):3575-3586 (1997). These authors conclude that, based on expression of this molecule, **heregulin** plays a role in vivo as a mesenchymal and neuronal factor. Similarly, Danilenko et al., Abstract 3101, FASEB 8(4-5):A535 (1994); Danilenko et al., Journal of Clinical Investigation 95(2): 842-851 (1995), found that the interaction of NDF and the **HER2** receptor is important in directing epidermal migration and differentiation during wound repair.

DETD [0057] "**Heregulin**" ligand is defined herein to be any isolated ligand, preferably a polypeptide sequence which possesses a biological property of a naturally occurring **heregulin** polypeptide that binds and activates **Her2**. Ligands within the scope of this invention include the **heregulin** polypeptides discussed in detail herein. **Heregulin** includes the polypeptides shown in FIGS. 1A-1D, 2A-2E, 3A-3E, 4A-4C, 5A-5D, 6A-6C, and 7A-7C and mammalian analogues thereof. Variants can. . .

DETD [0058] The term a "normal" hair **cell** or **inner-ear**-supporting **cell** means an hair **cell** or **inner-ear**-supporting **cell** which is not transformed, i.e., is non-cancerous and/or non-immortalized. Further, the normal hair **cell** or **inner-ear**-supporting **cell** is preferably not aneuploid. Aneuploidy exists when the nucleus of a **cell** does not contain an exact multiple of the haploid number of chromosomes, one or more chromosomes being present in greater or lesser number than the rest. Typical properties of transformed **cells** which fall outside the scope of this invention include the ability to form tumors when implanted into immune-deprived mice (nude mice), the ability to **grow** in suspension or in semi-solid media such as agar, a loss of contact inhibition allowing piling up of **cells** into colonies or foci, a loss of dependence on **growth** factors or serum, **cell** death if **cells** are inhibited from **growing**, and disorganization of actin filaments. Specifically included within the invention are normal **cells** which will not form tumors in mice, **grow** attached to plastic or glass (are anchorage dependent), exhibit contact inhibition, require serum-containing hormones and **growth** factors, remain viable if **growth** is arrested by

lack of serum, and contain well-organized actin filaments. Although the normal **inner-ear**-supporting **cells** are preferably not cultured **cells**, also suitable for the invention are non-transformed, non-immortalized epithelial **cells** isolated from mammalian tissue. These isolated **cells** may be cultured for several **generations** (up to about 10 or even 50 **generations**) in the presence of a **heregulin** in order to induce **growth** and/or **proliferation** of the isolated **inner ear** supporting **cell** sample, that is, to expand the sample. The expanded sample can then be reintroduced into the mammal for the purpose of repopulating the hair **cell** or **inner-ear**-supporting **cell** tissue (re-epithelialization). This is particularly useful for repairing tissue injury or damage.

DETD . . . purposes herein means an in vivo biologic or antigenic function or activity that is directly or indirectly performed by an **heregulin** sequence (whether in its native or denatured conformation), or by any subsequence thereof. Biologic functions include receptor binding, any enzyme. . . i.e. possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring **heregulin** polypeptide.

DETD [0060] "Biologically active" **heregulin** is defined herein as a polypeptide sharing a biologic function of an **heregulin** sequence which may (but need not) in addition possess an antigenic function. A principal known effect or function of **heregulin** is as a ligand polypeptide having a qualitative biological activity of binding to **HER2** resulting in the activation of the receptor tyrosine kinase (an "activating ligand"). One test for activating ligands is the **heregulin** tyrosine autophosphorylation assay described below. Included within the scope of **heregulin** as that term is used herein are **heregulin** having translated mature amino acid sequences of the complete human **heregulin** as set forth herein; deglycosylated or unglycosylated derivatives of **heregulin**, amino acid sequence variants of **heregulin** sequence, and derivatives of **heregulin**, which are capable of exhibiting a biological property in common with **heregulin**. While native **heregulin** is a membrane-bound polypeptide, soluble forms, such as those forms lacking a functional transmembrane domain, are also included within this definition. In particular, included are polypeptide fragments of **heregulin** sequence which have an N-terminus at any residue from about S216 to about A227, and its C-terminus at any residue. . .

DETD [0061] "Antigenically active" **heregulin** is defined as a polypeptide that possesses an antigenic function of an **heregulin** and which may (but need not) in addition possess a biologic function.

DETD [0062] In preferred embodiments, antigenically active **heregulin** is a polypeptide that binds with an affinity of at least about 10.sup.-9 I/mole to an antibody raised against a naturally occurring **heregulin** sequence. Ordinarily the polypeptide binds with an affinity of at least about 10.sup.-8 I/mole. Most preferably, the antigenically active **heregulin** is a polypeptide that binds to an antibody raised against one of heregulins in its native conformation. **Heregulin** in its native conformation generally is **heregulin** as found in nature which has not been denatured by chaotropic agents, heat or other treatment that substantially modifies the three dimensional structure of **heregulin** as determined, for example, by migration on nonreducing, nondenaturing sizing gels. Antibody used in this determination may be rabbit polyclonal antibody

raised by formulating native **heregulin** from a non-rabbit species in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of anti-**heregulin** antibody plateaus.

DETD [0063] Ordinarily, biologically or antigenically active **heregulin** will have an amino acid sequence having at least 75% amino acid sequence identity with a given **heregulin** sequence, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to an **heregulin** sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with **heregulin** residues in the **heregulin** of FIG. 6A-6C, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not. . . any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into **heregulin** sequence shall be construed as affecting homology.

DETD [0064] Thus, the biologically active and antigenically active **heregulin** polypeptides that are the subject of this invention include each entire **heregulin** sequence; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues from **heregulin** sequence; amino acid sequence variants of **heregulin** sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, **heregulin** sequence or its fragment as defined above; amino acid sequence variants of **heregulin** sequence or its fragment as defined above has been substituted by another residue. **heregulin** polypeptides include those containing predetermined mutations by, e.g., site-directed or PCR mutagenesis, and other animal species of **heregulin** polypeptides such as rabbit, rat, porcine, non-human primate, equine, murine, and ovine **heregulin** and alleles or other naturally occurring variants of the foregoing and human sequences; derivatives of **heregulin** or its fragments as defined above wherein **heregulin** or its fragments have been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope); glycosylation variants of **heregulin** (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of appropriate amino acid); and soluble forms of **heregulin**, such as HRG-GFD or those that lack a functional transmembrane domain.

DETD [0065] "Isolated" means a ligand, such as **heregulin**, which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for **heregulin**, and may include proteins, hormones, and other substances. In preferred embodiments, **heregulin** will be purified (1) to greater than 95% by weight of protein as determined by the Lowry method or other. . . marketed on the filing date hereof, or (3) to homogeneity by SDS-PAGE using Coomassie blue or, preferably, silver stain. Isolated **heregulin** includes **heregulin** in situ within recombinant cells since at least one component of **heregulin** natural environment will not be present. Isolated **heregulin** includes **heregulin** from one species in a recombinant cell culture of another species since **heregulin** in such circumstances will be devoid of source polypeptides. Ordinarily,

however, isolated **heregulin** will be prepared by at least one purification step.

DETD [0066] In accordance with this invention, **heregulin** nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active **heregulin**, is complementary to nucleic acid sequence encoding such **heregulin**, or hybridizes to nucleic acid sequence encoding such **heregulin** and remains stably bound to it under stringent conditions.

DETD [0067] Preferably, **heregulin** nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with an **heregulin** sequence. Preferably, the **heregulin** nucleic acid that hybridizes contains at least 20, more preferably at least about 40, and most preferably at least about. . .

DETD [0068] Isolated **heregulin** nucleic acid includes a nucleic acid that is identified and separated from at least one containment nucleic acid with which it is ordinarily associated in the natural source of **heregulin** nucleic acid. Isolated **heregulin** nucleic acid thus is present in other than in the form or setting in which it is found in nature. However, isolated **heregulin** encoding nucleic acid includes **heregulin** nucleic acid in ordinarily **heregulin**-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature. Nucleic acid encoding **heregulin** may be used in specific hybridization assays, particularly those portions of **heregulin** encoding sequence that do not hybridize with other known DNA sequences. "Stringent conditions" are those that (1) employ low ionic. . .

DETD [0081] The "**heregulin** tyrosine autophosphorylation assay" to detect the presence or bioactivity of **heregulin** ligands can be used to monitor the purification of a ligand for the **HER2** receptors. This assay is based on the assumption that a specific ligand for the receptor will stimulate autophosphorylation of the. . . receptor autophosphorylation. See Sadich et al., Anal. Biochem. 235:207-214 (1996). MDA-MB453 cells or MCF7 cells which contain high levels of p185.sup.**HER2** receptors but negligible levels of human EGF receptors, were obtained from the American Type Culture Collection, Rockville, Md. (ATCC No. . . gels were developed using the PROTOBLOT System from Promega. After drying the membranes, the density of the bands corresponding to p185.sup.**HER2** in each sample lane was quantitated with a Hewlett Packard SCANJET Plus Scanner attached to a Macintosh computer. The number of receptors per cell in the MDA-MB453 cells is such that under these experimental conditions the p185.sup.**HER2** receptor protein is the major protein which is labeled.

DETD [0097] II. Use and Preparation of **Heregulin** Sequences

DETD [0098] H. Preparation of **Heregulin** Sequences, Including Variants

DETD [0099] The system to be employed in preparing **heregulin** sequence will depend upon the particular **heregulin** sequence selected. If the sequence is sufficiently small **heregulin** may be prepared by in vitro polypeptide synthetic methods. Most commonly, however, **heregulin** will be prepared in recombinant cell culture using the host-vector systems described below. Suitable **heregulin** includes any biologically active and antigenetically active **heregulin**.

DETD . . . In general, mammalian host cells will be employed, and such

hosts may or may not contain post-translational systems for processing **heregulin** preprosequences in the normal fashion. If the host cells contain such systems then it will be possible to recover natural. . . vitro method. However, it is not necessary to transform cells with the complete prepro or structural genes for a selected **heregulin** when it is desired to only produce fragments of **heregulin** sequences. For example, a start codon is ligated to the 5' end of DNA encoding an HRG-GFD, this DNA is. . .

DETD [0101] **Heregulin** sequences located between the first N-terminal mature residue and the first N-terminal residue of HRG-GFD sequence, termed HRG-NTD, may function. . . molecule but from expression of DNA in which a stop codon is located at the C-terminus of HRG-NTD. In addition, **heregulin** variants are expressed from DNA encoding protein in which both the GFD and NTD domains are in their proper orientation. . .

DETD [0102] A preferred HRG-.alpha.ligand with binding affinity to p185.sup. **HER2** comprises amino acids 226-265 of FIG. 1A-D. This HRG-.alpha. ligand further may comprise up to an additional 1-20 amino acids. . . preceding amino acid 226 and 1-20 amino acids following amino acid 265. A preferred HRG-.beta. ligand with binding affinity to p185.sup.**HER2** comprises amino acids 226-265 of FIG. 2A-E. This HRG-.beta. ligand may comprise up to an additional 1-20 amino acids preceding. . .

DETD [0103] As noted above, other **heregulin** sequences to be prepared in accordance with this invention are those of the GFD. These are synthesized in vitro or. . . in recombinant cell culture. These are produced most inexpensively in yeast or E.coli by secretion under the control of a **heregulin**-heterologous signal as described infra, although preparation in mammalian cells is also contemplated using a mammalian protein signal such as that of tPA, UK or a secreted viral protein. The GFD can be the sequence of a native **heregulin** or may be a variant thereof as described below. GFD sequences include those in which one or more residues from. . .

DETD [0104] An additional **heregulin** is one which contains the GFD and the sequence between the C-terminus of GFD and the N-terminus of the transmembrane. . . C-terminal cleavage domain or CTC). In this variant (HRG-GFD-CTC) the DNA start codon is present at the 5' end of **heregulin**-heterologous signal sequence or adjacent the 5' end of the GFD-encoding region, and a stop codon is found in place of. . .

DETD [0106] If it is desired to prepare the longer **heregulin** polypeptides and the 5' or 3' ends of the given **heregulin** are not described herein, it may be necessary to prepare nucleic acids in which the missing domains are supplied by homologous regions from more complete **heregulin** nucleic acids. Alternatively, the missing domains can be obtained by probing libraries using the DNAs disclosed in the Figures or. . .

DETD [0107] A. Isolation of DNA Encoding **Heregulin**

DETD [0108] The DNA encoding **heregulin** may be obtained from any cDNA library prepared from tissue believed to possess **heregulin** mRNA and to express it at a detectable level. HRG-.alpha. gene thus may be obtained from a genomic library. Similar procedures may be used for the isolation of other **heregulin**, such as HRG-.beta.1, HRG-.beta.2, or HRG-.beta.3 encoding genes.

DETD . . . preferably human breast, colon, salivary gland, placental, fetal, brain, and carcinoma cell lines. Other biological sources of DNA encoding an **heregulin**-like ligand include other mammals and birds. Among the preferred mammals are members of the following orders: bovine, ovine, equine, murine,. . .

DETD [0118] B. Amino Acid Sequence Variants of **Heregulin**

DETD [0119] Amino acid sequence variants of **heregulin** are prepared by introducing appropriate nucleotide changes into **heregulin** DNA, or by in vitro synthesis of the desired **heregulin** polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for human **heregulin** sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Excluded from the scope of this invention are **heregulin** variants or polypeptide sequences that are not novel and unobvious over the prior art. The amino acid changes also may. . . such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, altering the intra-cellular location of **heregulin** by inserting, deleting, or otherwise affecting the leader sequence of the native **heregulin**, or modifying its susceptibility to proteolytic cleavage.

DETD [0120] The **heregulin** sequence may be proteolytically processed to create a number of **heregulin** fragments. HRG-GFD sequences of HRG-.alpha. all contain the amino acid sequence between HRG-.alpha. cysteine 226 and cysteine 265. The amino. . .

DETD . . . fragment ligands of HRG-.beta.2 based upon the FIG. 3A-3E and HRG-.beta.3 based upon FIG. 4A-4C may be accomplished by cleaving **heregulin** sequences of FIGS. 3A-3E and 4A-4C preferably adjacent to an arginine, lysine, valine or methionine.

DETD [0122] In designing amino acid sequence variants of **heregulin**, the location of the mutation site and the nature of the mutation will depend on **heregulin** characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first. . .

DETD [0123] A useful method for identification of certain residues or regions of **heregulin** polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244:. . . a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the expressed **heregulin** variants are screened for the optimal combination of desired activity.

DETD . . . amino acid sequence variants: the location of the mutation site and the nature of the mutation. These are variants from **heregulin** sequence, and may represent naturally occurring alleles (which will not require manipulation of **heregulin** DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon **heregulin** characteristic to be modified. Obviously, such variations that, for example, convert **heregulin** into a known receptor ligand, are not included within the scope of this invention, nor are any other **heregulin** variants or polypeptide sequences that are not novel and unobvious over the prior art.

DETD . . . contiguous. Deletions may be introduced into regions of low homology with other EGF family precursors to modify the activity of **heregulin**. Deletions from **heregulin** in areas of substantial homology with other EGF family sequences will be more likely to modify the biological activity of **heregulin** more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of **heregulin** in the affected domain, e.g., cysteine crosslinking, beta-pleated sheet or alpha helix.

DETD . . . or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within **heregulin** sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, and most preferably 1 to 3. Examples of terminal insertions include **heregulin** with an N-terminal methionyl residue (an artifact of the direct expression of **heregulin** in bacterial recombinant cell culture), and fusion of a heterologous N-terminal signal sequence to the N-terminus of **heregulin** to facilitate the secretion of mature **heregulin** from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host. . .

DETD [0127] Other insertional variants of **heregulin** include the fusion to the N- or C-terminus of **heregulin** of an immunogenic polypeptide, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions of **heregulin**-ECD with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described. . .

DETD [0128] Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in **heregulin** molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of **heregulin**, and sites where the amino acids found in **heregulin** ligands from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. A likely sub-domain of HRG-GFD. . .

DETD [0129] Other sites of interest are those in which particular residues of **heregulin**-like ligands obtained from various species are identical. These positions may be important for the biological activity of **heregulin**. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a. . .

DETD [0130] Substantial modifications in function or immunological identity of **heregulin** are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone. . .

DETD . . . entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of **heregulin** that are homologous with other receptor ligands, or, more preferably, into the non-homologous regions of the molecule.

DETD [0140] Any cysteine residues not involved in maintaining the proper conformation of **heregulin** also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DETD [0152] Another **heregulin** variant is or gamma-**heregulin** . -HRG is any polypeptide sequence that possesses at least one biological property of native sequence -HRG having SEQ ID NO:11. The biological property of this variant is the same as for **heregulin** noted above. This variant encompasses not only the polypeptide isolated from a native -HRG source such as human MDA-MB-175 cells. . . residues within the amino acid sequence shown for the human protein in FIG. 7A-7C as generally described agove for other **heregulin**. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct. . .

DETD . . . the variant has an amino acid substitution at a selected residue corresponding to a residue of 645-amino acid native human

heregulin-.beta.1 selected from:

DETD [0156] Other **heregulin**-.beta.1 variants include an amino acid substitution selected from:

DETD [0158] In a variation of this embodiment, the **heregulin** variant includes sets of amino acid substitutions selected from this group.

DETD [0159] In addition to including one or more of the amino acid substitutions disclosed herein, the **heregulin** variant can have one or more other modifications, such as an amino acid substitution, an insertion of at least one. . . amino acid, a deletion of at least one amino acid, or a chemical modification. For example, the invention provides a **heregulin** variant that is a fragment. In a variation of this embodiment, the fragment includes residues corresponding to a portion of human **heregulin**-.beta.1 extending from about residue 175 to about residue 230 (i.e., the EGF-like domain). For example, the fragment can extend from. . .

DETD [0160] DNA encoding amino acid sequence variants of **heregulin** is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation. . . by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of **heregulin**. These techniques may utilize **heregulin** nucleic acid (DNA or RNA), or nucleic acid complementary to **heregulin** nucleic acid.

DETD [0161] Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of **heregulin** DNA. This technique is well known in the art as described by Adelman et al., DNA, 2: 183 (1983). Briefly, **heregulin** DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of **heregulin**. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in **heregulin** DNA.

DETD . . . DNA encodes the mutated form of heregutin, and the other strand (the original template) encodes the native, unaltered sequence of **heregulin**. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as E. coli JM101. After. . .

DETD [0166] DNA encoding **heregulin** mutants with more than one amino acid to be substituted may be generated in one of several ways. If the. . .

DETD [0169] PCR mutagenesis is also suitable for making amino acid variants of **heregulin**. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR. . .

DETD . . . technique described by Wells et al. (Gene, 34: 315, 1985). The starting material is the plasmid (or other vector) comprising **heregulin** DNA to be mutated. The codon(s) in **heregulin** DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified. . . restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in **heregulin** DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize. . . of the linearized plasmid, such that it can

be directly ligated to the plasmid. This plasmid now contains the mutated **heregulin** DNA sequence.

DETD [0180] The cDNA or genomic DNA encoding native or variant **heregulin** is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available,. . .

DETD [0182] In general, the signal sequence may be a component of the vector, or it may be a part of **heregulin** DNA that is inserted into the vector. The native **heregulin** DNA is believed to encode a signal sequence at the amino terminus (5' end of the DNA encoding **heregulin**) of the polypeptide that is cleaved during post-translational processing of the polypeptide to form the mature **heregulin** polypeptide ligand that binds to the **HER2/HER3** receptor, although a conventional signal structure is not apparent. Native **heregulin** is, secreted from the cell but remains lodged in the membrane because it contains a transmembrane domain and a cytoplasmic region in the carboxyl terminal region of the polypeptide. Thus, in a secreted, soluble version of **heregulin** the carboxyl terminal domain of the molecule, including the transmembrane domain, is ordinarily deleted. This truncated variant **heregulin** polypeptide may be secreted from the cell, provided that the DNA encoding the truncated variant encodes a signal sequence recognized. . .

DETD [0183] **Heregulin** of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a. . . polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of **heregulin** DNA that is inserted into the vector. Included within the scope of this invention are **heregulin** with the native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be. . . by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native **heregulin** signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II leaders. For yeast secretion the native **heregulin** signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the. . .

DETD . . . in Bacillus genomic DNA. Transfection of Bacillus with this vector results in homologous recombination with the genome and insertion of **heregulin** DNA. However, the recovery of genomic DNA encoding **heregulin** is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise **heregulin** DNA. DNA can be amplified by PCR and directly transfected into the host cells without any replication component.

DETD . . . example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up **heregulin** nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which. . . in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes **heregulin**. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of **heregulin** are synthesized from the amplified DNA.

DETD . . . of the DHFR gene, and, concomitantly, multiple copies of other

DNA comprising the expression vectors, such as the DNA encoding **heregulin**. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of. . . employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding **heregulin**, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in. . .

DETD . . . Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to **heregulin** nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as **heregulin** to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters. . . promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding **heregulin** by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native **heregulin** promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of **heregulin** DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed **heregulin** as compared to the native **heregulin** promoter.

DETD . . . are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding **heregulin** (Siebenlist et al., Cell 20: 269, 1980) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding **heregulin**.

DETD [0200] **Heregulin** gene transcription from vectors in mammalian host cells may be controlled by promoters obtained from the genomes of viruses such. . . mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with **heregulin** sequence, provided such promoters are compatible with the host cell systems.

DETD [0203] Transcription of a DNA encoding **heregulin** of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting. . . for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to **heregulin** DNA, but is preferably located at a site 5' from the promoter.

DETD . . . DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding **heregulin**. The 3' untranslated regions also include transcription termination sites.

DETD . . . the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding **heregulin**. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host. . . physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of **heregulin** that have **heregulin**-like activity. Such a transient expression system is described in U.S. Pat.

No. 5,024,939.

DETD [0209] Other methods, vectors, and host cells suitable for adaptation to the synthesis of **heregulin** in recombinant vertebrate cell culture are described in Gething et al., Nature 293: 620-625, 1981; Mantei et al., Nature, 281:. . . 1979; Levinson et al., EP 117,060 and EP 117,058. A particularly useful expression plasmid for mammalian cell culture expression of **heregulin** is pRK5 (EP pub. no. 307,247) or pSVI6B (U.S. Ser. No. 07/441,574, filed Nov. 22, 1989, the disclosure of which. . .

DETD [0212] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for **heregulin** -encoding vectors. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number. . .

DETD [0213] Suitable host cells for the expression of glycosylated **heregulin** polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any. . . cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens, which has been previously manipulated to contain **heregulin** DNA. During incubation of the plant cell culture with A. tumefaciens, the DNA encoding **heregulin** is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express **heregulin** DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and. . .

DETD [0219] Prokaryotic cells used to produce **heregulin** polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

DETD [0220] The mammalian host cells used to produce **heregulin** of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal. . .

DETD [0222] It is further envisioned that **heregulin** of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding **heregulin** currently in use in the field. For example, a powerful promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element. . . genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired **heregulin**. The control element does not encode **heregulin** of this invention, but the DNA is present in the host cell genome. One next screens for cells making **heregulin** of this invention, or increased or decreased levels of expression, as desired.

DETD . . . either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native **heregulin** polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further below.

DETD [0227] G. Purification of The **Heregulin** Polypeptides

DETD [0228] **Heregulin** is recovered from a cellular membrane fraction. Alternatively, a proteolytically cleaved or a truncated expressed soluble **heregulin** fragment or subdomain are recovered from the culture medium as a soluble polypeptide. A **heregulin** is recovered from host cell lysates when directly expressed without a secretory signal.

DETD [0229] When **heregulin** is expressed in a recombinant cell other than one of human origin, **heregulin** is completely free of proteins or polypeptides of human origin. However, it is desirable to

purify **heregulin** from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to **heregulin**. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. **Heregulin** is then be purified from both the soluble protein fraction (requiring the presence of a protease) and from the membrane fraction of the culture lysate, depending on whether **heregulin** is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation;. . .

DETD [0230] **Heregulin** variants in which residues have been deleted, inserted or substituted are recovered in the same fashion as the native **heregulin**, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a **heregulin** fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-**heregulin** column can be employed to absorb **heregulin** variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenylmethylsulfonylfluoride (PMSF) also may. . . to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native **heregulin** may require modification to account for changes in the character of **heregulin** variants or upon expression in recombinant cell culture.

DETD [0231] H. Covalent Modifications of **Heregulin**

DETD [0232] Covalent modifications of **heregulin** polypeptides are included within the scope of this invention. Both native **heregulin** and amino acid sequence variants of **heregulin** optionally are covalently modified. One type of covalent modification included within the scope of this invention is a **heregulin** polypeptide fragment. **Heregulin** fragments, such as HRG-GDF, having up to about 40 amino acid residues are conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length **heregulin** polypeptide or **heregulin** variant polypeptide. Other types of covalent modifications of **heregulin** or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of **heregulin** or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N-. . .

DETD [0239] Derivatization with bifunctional agents is useful for crosslinking **heregulin** to a water-insoluble support matrix or surface for use in a method for purifying anti-**heregulin** antibodies, and vice versa, Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid,. . .

DETD [0242] **Heregulin** optionally is fused with a polypeptide heterologous to **heregulin**. The heterologous polypeptide optionally is an anchor sequence such as that found in a phage coat protein such as M13 gene III or gene VIII proteins. These heterologous polypeptides can be covalently coupled to **heregulin** polypeptide through side chains or through the terminal residues.

DETD [0243] **Heregulin** may also be covalently modified by altering its native glycosylation pattern. One or more carbohydrate substitutents in these embodiments, are modified by adding, removing or varying the

monosaccharide components at a given site, or by modifying residues in **heregulin** as that glycosylation sites are added or deleted.

DETD [0245] Glycosylation sites are added to **heregulin** by altering its amino acid sequence to contain one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites).. . . alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to **heregulin** (for O-linked glycosylation sites). For ease, **heregulin** is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding **heregulin** at preselected bases such that codons are generated that will translate into the desired amino acids.

DETD [0246] Chemical or enzymatic coupling of glycosides to **heregulin** increases the number of carbohydrate substituents. These procedures are advantageous in that they do not require production of the polypeptide. . .

DETD [0247] Carbohydrate moieties present on an **heregulin** also are removed chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an. . . al. (Arch. Biochem. Biophys., 259:52 (1987)) and by Edge et al. (Anal. Biochem., 118:131 (1981)). Carbohydrate moieties are removed from **heregulin** by a variety of endo- and exo-glycosidases as described by Thotakura et al. (Meth. Enzymol., 138:350 (1987)).

DETD [0249] **Heregulin** may also be modified by linking **heregulin** to various nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos.. . .

DETD [0250] One preferred way to increase the in vivo circulating half life of non-membrane bound **heregulin** is to conjugate it to a polymer that confers extended half-life, such as polyethylene glycol (PEG). (Maxfield, et al, Polymer. . .

DETD [0251] **Heregulin** may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or. . .

DETD . . . in order to select the optimal variant for the purpose intended. For example, a change in the immunological character of **heregulin**, such as a change in affinity for a given antigen or for the **HER2** receptor, is measured by a competitive-type immunoassay using a standard or control such as a native **heregulin** (in particular native **heregulin**-GFD). Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability. . .

DETD [0266] Hybridoma cell lines producing antibodies are identified by screening the culture supernatants for antibody which binds to **HER2** and/or **HER3** receptors. This is routinely accomplished by conventional immunoassays using soluble receptor preparations or by FACS using celi-bound receptor and labeled candidate antibody. Agonist antibodies are preferably antibodies which stimulate autophosphorylation in the **heregulin** tyrosine autophosphorylation assay described above.

DETD . . . Natl. Acad. Sci., 81:6851 (1984); Neuberger et al., Nature 312:604 (1984); Takeda et al., Nature 314:452 (1985)) containing a murine anti-**HER2**/**HER3** variable region and a human constant region of appropriate biological activity (such as ability to activate human complement and mediate. . .

DETD [0271] The **heregulin** are used in the present invention to

induce **inner-ear**-supporting **cell proliferation** to enhance new hair **cell generation**. These effects allow treatment of disease states associated with tissue damage, for example, ototoxic injury, or acoustic assault, degenerative hearing. . .

DETD . . . The field of cochlear implantation has also provided insights into both the short- and long-term effects of cochlear fenestration on **inner ear** function. Administration of **growth** factors to the inner ears of animals is now possible with the use of implanted catheters and miniature infusion pumps. Localized application of **heregulin** to the human **inner ear** can be performed to treat **inner ear** disorders related to hair **cell** disfunction.

DETD [0273] Therapeutic formulations of **heregulin** or agonist antibody are prepared for storage by mixing the **heregulin** protein having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, supra), in. . .

DETD [0274] **Heregulin** or agonist antibody to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The **heregulin** or antibody ordinarily will be stored in lyophilized form or in solution.

DETD [0275] Therapeutic **heregulin** or antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag. . .

DETD [0276] The route of **heregulin** or antibody administration is in accord with known methods, e.g., injection or infusion administration to the inner ear, or intralesional routes, or by sustained release systems as noted below. The **heregulin** ligand may be administered continuously by infusion or by bolus injection. An agonist antibody is preferably administered in the same. . .

DETD [0277] The **heregulin**, **heregulin** variant or fragment and agonist antibodies may be spray dried or spray freeze dried using known techniques (Yeo et al,. . .

DETD [0279] Sustained-release **heregulin** or antibody compositions also include liposomally entrapped **heregulin** or antibody. Liposomes containing **heregulin** or antibody are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Nat. Acad. Sci. USA, 82:. . . which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal **heregulin** therapy. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

DETD . . . of infection of a mammal by administration of an aminoglycoside antibiotic, the improvement comprising administering a therapeutically effective amount of **heregulin** or agonist, to the patient in need of such treatment to reduce or prevent ototoxin-induced hearing impairment associated with the. . .

DETD [0282] Also provided herein are methods for promoting new **inner ear** hair **cells** by inducing **inner ear** supporting **cell proliferation**, **regeneration**, or **growth** upon, prior to, or after exposure to an agent or effect that is capable of inducing a hearing or balance. . . impairment or disorder. Such agents and effects are those described herein. The method includes the step of administering to the **inner ear** hair **cell** an effective amount of **heregulin** or agonist or factor disclosed herein as useful. Preferably, the method is used upon, prior to, or after exposure to. .

.

DETD [0287] The **heregulin** or agonist is directly administered to the patient by any suitable technique, including parenterally, intranasally, intrapulmonary, orally, or by absorption. . .

DETD [0288] The **heregulin** or antibody agonist, can be combined and directly administered to the mammal by any suitable technique, including infusion and injection.. . . of administration will depend, e.g., on the medical history of the patient, including any perceived or anticipated side effects using **heregulin** alone, and the particular disorder to be corrected. Examples of parenteral administration include subcutaneous, intramuscular, intravenous, intraarterial, and intraperitoneal administration.. . .

DETD [0290] An effective amount of **heregulin** or antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Also, the amount of **heregulin** polypeptide will generally be less than the amount of an agonist antibody. Accordingly, it will be necessary for the therapist. . . 1 mg/kg and up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer **heregulin** or antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored. . .

DETD [0291] In a further embodiment, **inner-ear**-supporting **cells** may be obtained or isolated from a mammalian tissue to obtain a normal **inner-ear**-supporting **cell** sample using techniques well known in the art (biopsy, etc.). This sample may then be treated with a **heregulin** protein in order to induce hair **cell** or **inner-ear** -supporting **cell growth** and/or **proliferation** in the sample thereby expanding the population of **inner-ear**-supporting **cells**. Typically, **heregulin** will be added to the in vitro **inner-ear**-supporting **cell** culture at a concentration of about 0.1 to about 100 nM preferably 1-50 nM. If desired, the primary **inner-ear** -supporting **cells** may be cultured in vitro for several **generations** in order to sufficiently expand the hair **cell** or **inner-ear**-supporting **cell** population. The hair **cell** or **inner-ear** -supporting **cells** are cultured under conditions suitable for mammalian **cell** culture as discussed above. After expansion, the expanded sample is reintroduced into the mammal for the purpose of re-epithelializing the. . .

DETD [0292] The methods and procedures described herein with respect to HRG-.alpha. or **heregulin** in general may be applied similarly to other **heregulin** such as HRG-.beta.1, HRG-.beta.2 and HRG-.beta.3 and to variants thereof, as well as to the antibodies. All references cited in. . .

DETD [0293] Characterization of **Inner-Ear**-Supporting **Cell** Cultures

DETD [0299] A much greater number of BrdU-positive cells were seen in the cultures containing **heregulin** (HRG-.beta.1-177-244) than any of the other factors known to activate Her receptors. Cell counts performed from the control cultures and cultures containing confirmed that **heregulin** significantly enhanced proliferation of the utricular supporting cells ($p<0.0001$, FIG. 9). IGF-1 at 100 nM, TGF-.alpha. at 100 nM (R. . . et al., EMBO Journal 16(6):1268-78 (1997)), and IGF1-binding protein at 100 nM were weaker mitogens, if at all, compared to **heregulin**. SMDF polypeptides are prepared as

described in WO 96/15244. Neuregulin-3, a neural tissue-enriched protein that binds and activates erbB4, was. . .

DETD [0300] To determine whether the effect of **heregulin** was dose-dependent, a dose-dependent study was carried out in the utricular epithelial sheet cultures at a range of 0.03 nM to 10 nM **heregulin** (FIG. 10). A **heregulin**-dose-dependent increase in the number of BrdU positive cells was observed. Maximal effect of **heregulin** was seen at 3 nM.

DETD . . . in Zheng et al. (Journal of Neuroscience, 17(21):8270-82 (1997)). This system provides an excellent means to test the effect of **heregulin** on supporting cell proliferation in a physiologically significant system that mimics the in vivo state. In particular, the effects of **heregulin** after ototoxic-induced damage (e.g. antibiotic gentamycin) were examined.

DETD . . . mounts were cultured 1-2 days after explant, then treated with gentamycin (1 mM) for two days, and then treated with **heregulin** (3 nM) for 11 days in the presence of tritiated thymidine. To determine the number of labeled **cells**, the tissue was fixed, sectioned and processed for autoradiography. In response to **heregulin**, compared to control cultures, an increase in the number of .sup.3H-thymidine labeled **cells** in both the supporting **cell** layer (SC) and the hair **cell** layer (HC) was observed as shown in FIGS. 11A-D, which represent similarly treated samples. The **cell** count of .sup.3H-thymidine labeled **cells** in both the supporting **cell** layer and in the hair **cell** layer increased significantly compared to control cultures lacking **heregulin** as shown in FIG. 12. The data is consistent with the data obtained in the utricular sheet cultures. And the data indicates that **heregulin** can act to increase **inner-ear**-supporting **cell** **proliferation**, which leads to hair **cell** **generation**, in instances following hair **cell** damage and injury.

DETD [0306] **Heregulin** Acts through the **Her2** Receptor

DETD [0307] To provide further evidence that **heregulin** is a physiologically relevant factor and that it acts through a physiologically relevant receptor, the mRNA expression levels of **heregulin** and its receptors **Her2**, **Her3** and Her4 in the hair **cell** and supporting **cell** layers of the rat utricular sensory epithelium were determined. RNA was extracted from the P3 utricle sheet cultures and also from UEC4 **cells** (a **inner-ear**-supporting **cell** line). Using TaqMan PCR analysis with appropriate gene-specific primers (Heid et al., Genome Research. 6(10):986-94 (1996)), it was observed that all four were expressed in the **inner ear**, however, **heregulin** and **Her2** were expressed at a higher level than either **Her3** or Her4 (see FIG. 13). Her4 was not expressed in the **inner-ear**-supporting **cell** line.

DETD [0308] To determine that **Her2** was indeed expressed at the protein level and to confirm its localization, fluorescently labeled anti-**Her2** monoclonal antibody was used to immunostain rat P0 (day zero) cochlea and adult utricle. **Her2** was localized to the hair **cell** and supporting **cell** sensory epithelium layers in the **inner ear** (see FIG. 14 A (cochlea) and FIG. 14B (utricle)). Anti-**HER2** monoclonal antibodies 2C4 and 4D5 have been described elsewhere (Fendly et al. Cancer Research 50:1550-1558 (1990)). Consistent with this observation is that immunostaining with a **heregulin** antibody suggests that

heregulin is expressed by hair **cells** of the **inner ear**.

DETD [0309] Addition of neutralizing monoclonal antibodies against **Her2**, but not the addition of the immunoadhesin Her4-IgG, at saturating amounts to the utricular cultures, blocked the effects of **heregulin**. Thus, **heregulin** stimulates supporting cell proliferation and hence the generation of new hair cells by activating a **Her2**-mediated signaling pathway, but not a Her4-modiated pathway.

DETD [0310] In addition, preliminary experiments with embryonic rat **inner ear** explant cultures show that **heregulin** affects hair **cell** differentiation by enhancing **proliferation** of hair **cell** progenitors. Rat E14 otocyst cultures treated with **heregulin** respond with an increase in the number of hair **cell** progenitor **cells** compared to untreated cultures. This is consistent with the adult tissue studies, indicating that **heregulin** stimulates the **proliferation** of **cells** that differentiate into hair **cells**.

DETD [0311] **Heregulin** Acts In Vivo to Enhance **Inner Ear** Supporting **Cell Proliferation** and Hair **Cell Generation** Following Ototoxic Injury and Acoustic Assault

DETD [0312] Chinchillas are an accepted model to test the effects of factors and agents against or following hair **cell** damage or injury. Chinchillas can be treated with gentamicin, caboplatin or acoustic trauma. Preferably, at least five chinchillas are in. . . assault and allowed to recover. Typically, four to six weeks is sufficient for recovery. The test group is treated with **heregulin** in addition to the injury. All animals will receive BrdU, preferably subcutaneous infusion, using minipumps, to label the dividing **cells** during the treatment period. **Heregulin**, or one of the **heregulin** factors as taught herein, will be administered to the **inner ear**. Minipumps can be used. The **heregulin** can be infused into the cochlea. After the treatment period, cochlea and utricular maculae are dissected out of the animals. The tissue is fixed and BrdU immunohistochemical labeling done. BrdU labeled **cells** in the **inner ear** sensory epithelium are counted. **Cell** counts from the two groups--are compared and analyzed statistically to determine the amount of enhancement of **proliferation** of supporting **cells** and new hair **cell generation** induced by the **heregulin** treatment.

DETD [0532] Forge A, Li L, Corwin J T, Nevill G (1993) Ultrastructural evidence for hair **cell regeneration** in the mammalian **inner ear**. Science 259:1616-1619.

DETD [0568] Lambert P R (1994) **Inner ear** hair **cell regeneration** in a mammal: identification of a triggering factor. Laryngoscope 104:701-718.

DETD [0606] Tsue T T, Oesterle E C, Rubel E W (1994a) Diffusible factors regulate hair **cell regeneration** in the avian **inner ear**. Proc Natl Acad. Sci USA 91:1584-1588.

DETD [0607] Tsue T T, Oesterle E C, Rubel E W (1994b) Hair **cell regeneration** in the **inner ear**. Otolaryngol. Head Neck Surg 111:281-301.

DETD . . . S., Patterson, S. L., Heuer, J. G., Wheeler, E. F., Bothwell, M., and Rubel, E. W. (1991). Expression of nerve **growth** factor (NGF) receptors in the developing **inner ear** of chick

and rat. Development 113: 455-470.

DETD [0612] Warchol, M. E., Lambert, P. R., Goldstein, B. J., Forge, A., and Corwin, J. T. (1993). **Regenerative proliferation** in **inner ear** sensory epithelia from adult Guinea pigs and humans. Science 259:1619-1622.

DETD [0618] Yamashita H, Oesterle E C (1995) Induction of **cell proliferation** in mammalian **inner-ear** sensory epithelia by transforing **growth** factor a and epidermal **growth** factor. Proc Natl Acad Sci USA 92:3152-3155.

CLM What is claimed is:

1. A method of inducing hair **cell generation** or **inner-ear**-supporting **cell growth**, **regeneration**, and/or **proliferation**, comprising contacting an **inner-ear**-supporting **cell** which expresses **HER2** and/or **HER3** receptors with an effective amount of an isolated ligand which activates **HER2** and/or **HER3** receptors or a combination thereof.

2. The method of claim 1, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the **HER2** or **HER3** receptor.

3. The method of claim 2, wherein the activating ligand is human **heregulin** or a fragment thereof.

6. The method of claim 2, wherein the activating ligand is recombinant human **heregulin** or a fragment thereof.

11. The method of claim 6, wherein the **heregulin** is rHRG-.beta.1-177-244.

12. The method of claim 1, wherein the **inner-ear** -supporting **cell** is in the utricle or cochlea.

13. The method of claim 1 wherein the **inner-ear** -supporting **cell** expresses **HER2**, **HER3**, or both.

14. A method of increasing the number of **inner ear** supporting **cells**, comprising administering to a patient in need thereof an effective amount of an isolated **HER2** and/or **HER3** activating ligand.

15. The method of claim 14, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the **HER2** and/or **HER3** receptor.

. . . a hair cell related hearing disorder, comprising administering to a patient in need thereof an effective amount of an isolated **HER2** and/or **HER3** activating ligand.

17. The method of claim 16, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the **HER2** and/or **HER3** receptor.

18. A method, comprising the steps of: (a) obtaining an **inner**-**ear**-supporting **cell** sample from a mammal; (b) contacting the sample with a ligand which activates **HER2** or **HER3** or a combination thereof to induce **growth** and/or **proliferation** of **inner**-**ear**-supporting **cells** in the sample and to obtain an expanded sample; and (c) re-introducing the expanded sample into the mammal.

L4 ANSWER 3 OF 3 USPATFULL

AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. A monoclonal antibody against adult rat utricular epithelium is also described.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

ACCESSION NUMBER:	2000:80853 USPATFULL
TITLE:	Monoclonal antibody against utricular epithelium
INVENTOR(S):	Carnahan, Josette F., Newbury Park, CA, United States
PATENT ASSIGNEE(S):	Amgen Inc., Thousands Oaks, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6080845		20000627
APPLICATION INFO.:	US 1999-238182		19990128 (9)
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1998-129549, filed on 5 Aug 1998, now abandoned		
DOCUMENT TYPE:	Utility		
FILE SEGMENT:	Granted		
PRIMARY EXAMINER:	Huff, Sheela		
LEGAL REPRESENTATIVE:	Mazza, Richard J., Levy, Ron K., Odre, Steven M.		
NUMBER OF CLAIMS:	1		
EXEMPLARY CLAIM:	1		
NUMBER OF DRAWINGS:	6 Drawing Figure(s); 5 Drawing Page(s)		
LINE COUNT:	672		

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. A monoclonal antibody against adult rat utricular epithelium is also described.

SUMM This invention relates to the NDF/**heregulin** protein family, and more specifically to the use of a derivative peptide to stimulate the **proliferation** of sensory epithelial **cells** of the **inner ear** for the treatment of vestibular disorders. The invention also relates to monoclonal antibodies raised against adult rat utricular epithelium.

SUMM The NDF/heregulins are a known family of molecules which stimulate the tyrosine phosphorylation of the erbB2/**Her2** protooncogene product p185; see Peles et al., Cell, Volume 69, pages 1-14 (1992); Wen et al., Cell, Volume 69, pages. . . 1205-1210 (1992); and Bacus et al., Cancer Research, Volume 53, pages 5251-5261 (1993). Thought at first to be ligands for erbB2/**Her2**, the NDF/heregulins are now known to bind to and stimulate the kinase activity of erbB3/**Her3** and erbB4/Her4; see Plowman et al., Nature, Volume 366, pages 473-475 (1993); Kita et al., FEBS Letters, Volume 349, pages 139-143 (1994); and

Carraway et al., Journal of Biological Chemistry, Volume 269, pages 14303-14306 (1994). The NDF/**heregulin** family is considered to also include ARIA and glial growth factor (GGF); see, respectively, Falls et al., Cell, Volume 72,. . .

SUMM . . . the EGF-like domain of NDF-.alpha.2 and is produced by chemical synthesis. The peptide is shown to stimulate tyrosine phosphorylation of **Her2**, **Her3** and Her4, and to induce morphological changes in breast cancer cells.

SUMM The present invention comprises the use of a peptide of following sequence as a **growth** stimulant for sensory epithelial **cells** of the **inner ear**:

SUMM . . . a hybrid form derived from the EGF-like domains of NDF-.alpha. and NDF-.beta.. However, the usefulness of this molecule as a **growth** stimulant for sensory epithelial **cells** of the utricle in the **inner ear**, which is demonstrated in the working examples below, has not been previously recognized. Because all of the vestibular organs (e.g.,. . . peptide may also be useful to treat hearing loss in mammals, including humans, which is attributable to the degeneration of **inner ear** hair **cells**, i.e., by **regenerating** such hair **cells** in association with sensory epithelium.

DRWD . . . a graph comparing the mitogenic activity (as BrdU-positive nuclei) of the peptide of SEQ ID NO: 1 ("Peptide") with other NDF/**heregulin**-derived peptides on **inner ear** sensory epithelial **cells**.

DETD The mitogenic activity of the peptide of SEQ ID NO: 1 on the vestibular sensory epithelium of the mammalian **inner ear** suggests that it may also be useful to **regenerate** hair **cells**, which are critical for hearing. Thus, the peptide may be beneficial for treating hearing loss associated with deteriorated or damaged **inner ear** hair **cells**, and such applications are included within the therapeutic treatments made possible by the present invention.

DETD Sensory epithelial **cells** obtained from utricles in the **inner ear** of both seven day-old (infant) rats and six week-old (adult) rats were isolated with the use of thermolysin treatment; see. . . page 87 (1995). All edges were trimmed away and the central portion of the epithelium was cut into quarters. Epithelial **cells** from the infant rats were cultured in DMEM/F12 with 10% FBS (Gibco BRL, Grand Island, N.Y.), and 3 micrograms per. . . 1 or 50 ng/ml of recombinant derived FGF-10, recombinant derived FGF-16, recombinant derived ciliary-derived neurotrophic factor (CNTF), recombinant derived neurotrophic **growth** factor (NGF), recombinant derived glial-derived neurotrophic factor (GDNF), recombinant derived keratinocyte **growth** factor (KGF), or a control (no **growth** factor present). The experiment was ended by fixing in 4% paraformaldehyde for one hour.

DETD Using the test procedure of Example 1, the peptide of SEQ ID NO: 1 was compared with members of the NDF-**heregulin** family in primary cultures of young rat utricular sensory epithelial cells, at a treatment concentration of 50 ng/ml in each. . .

DETD **Generation** of Monoclonal Antibodies Against Sensory Epithelial **Cells** of Rodent **Inner Ear**

DETD The lack of a specific marker for sensory epithelium **cells** adds to the challenges associated with research on hair **cell regeneration** in the **inner ear** of mammals. Monoclonal antibodies against hair **cells** have been reported in the literature; Finley et al., Assoc. Res. Otolaryngol. Abstr., Volume

20, page 16 (1997) and Holley et al., J. Neurocytol., Volume 24, pages 853-864 (1997). However, none of these antibodies are specific to supporting **cells** in the mammalian vestibular organs.

DETD . . . is a description of the preparation of four distinct monoclonal antibodies raised against rat utricular epithelia which specifically label supporting **cells** of the vestibular organs in the **inner ear** of the rodent. These antibodies constitute an additional aspect of the present invention.

DETD In this method, sensory epithelia were isolated from adult rat **inner ear** utricles by the thermolysin method; see above for description. Seventy pieces of epithelia were homogenized by ultrasound, and then emulsified. . . showed high titer against the antigen (i.e., the utricle extract). Mouse splenocytes were harvested and then fused with HL-1 myeloma **cells** (Kohler and Milstein, Nature, Volume 256, pages 495-497 (1975). Screening for monoclonal antibodies was conducted by immunostaining on frozen 10-micron. . .

DETD Each of the monoclonal antibodies specifically stained the supporting **cells**, but with a characteristically different pattern. SC-1 stained the top portion of the supporting **cells** brightly, while gradually decreasing around the **cell** nuclei. SC-2 stained only the top portion of the supporting **cells**. SC-3 immunoreactivity was concentrated on the lower cytoplasmic portion of the supporting **cells** in neotal rat utricles, and migrated to the upper portion in adult utricles. SC-4 immunoreactivity was found mostly in the supporting **cell** apex of the adult utricle. SC-4 and SC-3 immunostaining was found in embryonic progenitors of supporting **cells** of the **inner ear**.

=>

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FILE 'CAPLUS' ENTERED AT 11:48:13 ON 05 DEC 2002
USE IS SUBJECT TO THE TERMS OF YOUR STN CUSTOMER AGREEMENT.
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FILE 'USPATFULL' ENTERED AT 11:48:13 ON 05 DEC 2002
CA INDEXING COPYRIGHT (C) 2002 AMERICAN CHEMICAL SOCIETY (ACS)

=> s (heregulin or HRG(2a)(alpha? or beta?) or rHRG or recombinat(3a)heregulin?)
L5          996 (HEREGULIN OR HRG(2A)(ALPHA? OR BETA?) OR RHRG OR RECOMBINAT(3A)
                HEREGULIN?)

=> d his

     (FILE 'HOME' ENTERED AT 11:00:58 ON 05 DEC 2002)

     FILE 'CAPLUS, USPATFULL' ENTERED AT 11:01:45 ON 05 DEC 2002
L1           976 S HEREGULIN
L2             7 S L1 AND INNER(4A)EAR(P)(CELL# OR GROW? OR GENERAT? OR REGENERA
L3             6 DUP REM L2 (1 DUPLICATE REMOVED)

     FILE 'STNGUIDE' ENTERED AT 11:09:18 ON 05 DEC 2002

     FILE 'CAPLUS, USPATFULL' ENTERED AT 11:13:13 ON 05 DEC 2002
L4             3 S L3 AND (HER2 OR HER3)

     FILE 'STNGUIDE' ENTERED AT 11:21:42 ON 05 DEC 2002

     FILE 'STNGUIDE' ENTERED AT 11:27:25 ON 05 DEC 2002

     FILE 'CAPLUS, USPATFULL' ENTERED AT 11:48:13 ON 05 DEC 2002
L5           996 S (HEREGULIN OR HRG(2A)(ALPHA? OR BETA?) OR RHRG OR RECOMBINAT(

=> s l5 and inner(4a)ear(p)(cell# or grow? or generat? or regenerat? or proliferat?
or disorder?)
L6             8 L5 AND INNER(4A) EAR(P)(CELL# OR GROW? OR GENERAT? OR REGENERAT
                ? OR PROLIFERAT? OR DISORDER?)

=> dup rem l6
PROCESSING COMPLETED FOR L6
L7             7 DUP REM L6 (1 DUPLICATE REMOVED)

=> d l7 abs ibib kwic 1-7

L7   ANSWER 1 OF 7  USPATFULL
AB     Ligands which bind to the HER2 and/or HER3 receptors are useful as
       inner-ear-supporting cell growth
       factors to enhance proliferation-mediated generation
       of new hair cells.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.
ACCESSION NUMBER:       2002:156704  USPATFULL
TITLE:                  Hair cell disorders
INVENTOR(S):            Gao, Wei-Qiang, Foster City, CA, UNITED STATES

                             NUMBER       KIND    DATE
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09/849,868

PATENT INFORMATION:	US 2002081299	A1	20020627
APPLICATION INFO.:	US 2001-849868	A1	20010504 (9)

	NUMBER	DATE
PRIORITY INFORMATION:	US 1998-107522P	19981107 (60)

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: KNOBBE MARTENS OLSON & BEAR LLP, 620 NEWPORT CENTER DRIVE, SIXTEENTH FLOOR, NEWPORT BEACH, CA, 92660
NUMBER OF CLAIMS: 18
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 34 Drawing Page(s)
LINE COUNT: 5225
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Ligands which bind to the HER2 and/or HER3 receptors are useful as **inner-ear**-supporting **cell growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**.

SUMM [0002] This application relates to inducing, promoting, or enhancing the **growth**, **proliferation**, repair, **generation**, or **regeneration** of **inner ear** tissue, particularly **inner ear** epithelial hair **cells** and supporting **cells**. More particularly, this application relates to potently stimulating supporting **cell proliferation** and enhancing **proliferation**-mediated **generation** of new hair **cells**. In addition, this application provides methods, compositions and devices for prophylactic and therapeutic treatment of **inner ear disorders** and conditions, particularly sensorineural hearing and balance impairments. This invention relates to the use of HER2 ligands, in particular **heregulin** polypeptides, as **inner-ear**-supporting **cell growth** factors.

SUMM . . . a wide variety of causes, including infections, mechanical injury, loud sounds, aging, and chemical-induced ototoxicity that damages neurons and/or hair **cells** of the peripheral auditory system. The peripheral auditory system consists of auditory receptors, hair **cells** in the organ of Corti, and primary auditory neurons, the spiral ganglion neurons in the cochlea. Spiral ganglion neurons ("SGN") are primary afferent auditory neurons that deliver signals from the peripheral auditory receptors, the hair **cells** in the organ of Corti, to the brain through the cochlear nerve. The eighth nerve connects the primary auditory neurons. . . . are primary afferent sensory neurons responsible for balance and which deliver signals from the utricle, saccule and ampullae of the **inner ear** to the brain, to the brainstem. Destruction of primary afferent neurons in the spiral ganglia and hair **cells** has been attributed as a major cause of hearing impairments. Damage to the peripheral auditory system is responsible for a. . .

SUMM . . . nervous system may result in hearing loss or balance impairment. Auditory apparatus can be divided into the external and middle **ear**, **inner ear** and auditory nerve and central auditory pathways. While having some variations from species to species, the general characterization is common. . . . for all mammals. Auditory stimuli are mechanically transmitted through the external auditory canal, tympanic membrane, and ossicular chain to the **inner ear**. The middle **ear** and mastoid process are normally filled with air. **Disorders** of the

external and middle ear usually produce a conductive hearing loss by interfering with this mechanical transmission. Common causes. . . space. Auditory information is transduced from a mechanical signal to a neurally conducted electrical impulse by the action of neuro-epithelial **cells** (hair **cells**) and SGN in the **inner ear**. All central fibers of SGN form synapses in the cochlear nucleus of the pontine brain stem. The auditory projections from. . . to the auditory brain stem and the auditory cortex. All auditory information is transduced by a limited number of hair **cells**, which are the sensory receptors of the **inner ear**, of which the so-called inner hair **cells**, numbering a comparative few, are critically important, since they form synapses with approximately 90 percent of the primary auditory neurons.. . . nucleus, the number of neural elements involved is measured in the hundreds of thousands. Thus, damage to a relatively few **cells** in the auditory periphery can lead to substantial hearing loss or balance impairment. Hence, many causes of sensorineural loss can be ascribed to lesions in the **inner ear**. This hearing loss and balance impairment can be progressive. In addition, the hearing becomes significantly less acute because of changes. . .

SUMM [0007] The toxic effects of these drugs on auditory **cells** and spiral ganglion neurons are often the limiting factor for their therapeutic usefulness. For example, antibacterial aminoglycosides such as gentamicins,. . . infection has reached advanced stages, but at this point permanent damage may already have been done to the middle and **inner ear** structure. Clearly, ototoxicity is a dose-limiting side-effect of antibiotic administration. For example, nearly 75% of patients given 2 grams of. . .

SUMM [0009] Accordingly, there exists a need for means to prevent, reduce or treat the incidence and/or severity of **inner ear disorders** and hearing impairments involving **inner ear** tissue, particularly **inner ear** hair **cells**, and optionally, the associated auditory nerves. Of particular interest are those conditions arising as an unwanted side-effect of ototoxic therapeutic. . . a method that provides a safe, effective, and prolonged means for prophylactic or curative treatment of hearing impairments related to **inner ear** tissue damage, loss, or degeneration, particularly ototoxin-induced, and particularly involving **inner ear** hair **cells**. The present invention provides compositions and methods to achieve these goals and others as well.

SUMM [0011] In general an object of the invention is to provide a method of inducing, promoting, or enhancing the **growth**, **proliferation**, repair, or **regeneration** of **inner ear** tissue, particularly **inner ear** hair **cells** and their supporting **cells** for the purpose of promoting repair and healing of inner tissue damage or injury.

SUMM [0012] Accordingly, one object of this invention is to provide a method of treating **inner ear disorders** and conditions in patients, primarily human patients, in need of such treatment. A further object is to provide a method of inducing **inner-ear**-supporting **cell growth**, **generation**, and development, which leads to **generation** of new hair **cells**.

SUMM . . . this invention, it has now been discovered that these objects and the broader objective of treating conditions associated with hair **cell** or **inner-ear**-supporting **cell**

damage and injury are achieved by administering to a patient in need of such treatment an effective amount of a **heregulin** ligand, preferably a polypeptide or fragment thereof. These **heregulin** polypeptides, include **HRG**-.**alpha**., **HRG**-.**beta**.1, **HRG**-.**beta**.2, **HRG**-.**beta**.3 and other **heregulin** polypeptides which cross-react with antibodies directed against these family members and/or which are substantially homologous as defined below and includes **heregulin** variants such as N-terminal and C-terminal fragments thereof. A preferred **heregulin** is the ligand disclosed in FIG. 1A-1D and further designated **HRG**-.**alpha**.. Other preferred heregulins are the ligands disclosed in FIG. 2A-2E, and designated **HRG**-.**beta**.1; disclosed in FIG. 3A-3E designated **HRG**-.**beta**.2; and disclosed in FIG. 4A-4C designated **HRG**-.**beta**.3.

SUMM [0014] In another aspect, the invention provides a method in which **heregulin** agonist antibodies are administered to achieve the objects of the invention. In this embodiment, HER2/HER3 or fragments thereof (which also. . . HER3, preferably Her2. In addition, antibodies may be selected that are capable of binding specifically to individual family members of **heregulin** family, e.g. **HRG**-.**alpha**., **HRG**-.**beta**.1, **HRG**-.**beta**.2, **HRG**-.**beta**.3, and which are agonists thereof.

SUMM [0015] In general, the invention is a method of **regenerating** and/or repairing hair **cell** or **inner-ear**-supporting **cell** injury by stimulating **growth** and **proliferation** of **inner-ear**-supporting **cells** to enhance **generation** of new hair **cells**. The hair **cells** may be injured by many types of insults, for example, injury due to surgical incision or resection, chemical or smoke inhalation or aspiration, chemical or biochemical ulceration, **cell** damage due to viral or bacterial infection, etc The **inner-ear**-supporting **cells** which may be affected by the method of the invention include any **inner-ear**-supporting **cell** which expresses HER2 or HER3, preferably Her3. The method of the invention stimulates **growth** and **proliferation** of the **inner-ear**-supporting **cells** leading to **generation** of new hair **cells** to repair and re-establish the sensorineural contacts in the **inner ear** to allow the affected tissues to develop normal physiological functions more quickly.

SUMM [0016] Accordingly, one embodiment of the invention is a method of inducing **inner-ear**-supporting **cell** **growth** by contacting a **inner-ear**-supporting **cell** which expresses HER2 receptor with an effective amount of a HER2 activating ligand.

SUMM [0017] A further embodiment is a method of treating **inner ear** hair **cell** injury, caused by ototoxins or acoustic assault for example, by administering to a patient in need thereof an effective amount. . .

DRWD . . . sequence (SEQ ID NO:2) contained in a clone obtained according to U.S. Pat. No. 5,367,060. The initiating methionine (Met) of **HRG**-.**alpha**. is at position 45.

DRWD . . . sequence (SEQ ID NO:4) of a potential coding sequence of a clone obtained according to U.S. Pat. No. 5,367,060 for **HRG**-.**beta**.1. The initiating Met is at M31.

DRWD . . . cDNA sequence (SEQ ID NO:6) of a nucleotide sequence of a clone

obtained according to U.S. Pat. No. 5,367,060 for **HRG**-.
beta.2.

DRWD . . . cDNA sequence (SEQ ID NO:8) of a nucleotide sequence of a clone obtained according to U.S. Pat. No. 5,367,060 for **HRG**-.
beta.3.

DRWD . . . cDNA sequence (SEQ ID NO:10) of a nucleotide sequence of a clone obtained according to U.S. Pat. No. 5,367,060 for **HRG**-.
beta.2-like protein.

DRWD . . . .beta.2-like and .beta.3 in descending order and illustrates the amino acid insertions, deletions, and substitutions that characterize these forms of **heregulin** (SEQ ID NOS: 1, 3, 5, 9, and 7).

DRWD [0027] FIG. 10 shows the dose-dependent proliferation effect of **heregulin** on cells in the rat utricular sheet hair cell layer, as indicated by the number of BrdU positive cells per. . .

DRWD . . . 11A-D show autoradiography of tritiated-thymidine labeled cells in supporting cell and hair cell layer in gentamicin-treated utricles in response to **heregulin** treatment. FIGS. A-D are views from similarly treated organotypic rat utricular whole mounts.

DRWD . . . tritiated-thymidine labeled cells in supporting cell and hair cell layer in gentamicin-treated utricles (shown in FIGS. 11A-D) in response to **heregulin** treatment compared to control.

DRWD [0030] FIG. 13 shows the RNA concentration of **heregulin** and the recepters Her2, Her3 and Her4 in RAN isolated from the inner ear sensory epithelium layer.

DRWD [0031] FIG. 14 shows localization of Her2, a **heregulin** receptor, in the inner ear sensory epithelium, as indicated by immunostaining the P0 cochlea and adult utricle with labeled monoclonal. . .

DETD [0032] **Heregulin** ligands, in particular polypeptides and agonist antibodies thereof, have affinity for and stimulate the HER2, and less preferably HER3, receptors or combinations thereof in autophosphorylation. Included within the definition of **heregulin** ligands, in addition to **HRG**-.**alpha**., **HRG**-.
beta.1, **HRG**-.**beta**.2, **HRG**-.
beta.3 and **HRG**-.**beta**.2-like, are other polypeptides binding to the HER2 receptor, which bear substantial amino acid sequence homology to **HRG**-.**alpha**. or **HRG**
-.**beta**.1. Such additional polypeptides fall within the definition of **heregulin** as a family of polypeptide ligands that bind to the HER2 receptors.

DETD [0033] **Heregulin** polypeptides bind with varying affinities to the HER2 receptors. It is also known that heterodimerization of HER2 with HER3 occurs with subsequent receptor cross-phosphorylation as described above. In the present invention, **inner-ear**
-supporting **cell growth** and/or **proliferation** is induced when a **heregulin** protein interacts and binds with an individual receptor molecule or a receptor dimer such that receptor phosphorylation is induced. Binding. . .

DETD . . . superfamily and consists of three distinct receptors, HER2, HER3, and HER4. A ligand for this ErbB family is the protein **heregulin** (HRG), a multidomain containing protein with at least 15 distinct isoforms.

DETD [0039] The quest for the activator of the HER2 oncogene has lead to the discovery of a family of **heregulin** polypeptides. These proteins appear to result from alternate splicing of a single gene which was mapped to the short arm. . .

DETD [0040] Holmes et al. isolated and cioned a family of polypeptide

activators for the HER2 receptor which they called **heregulin**-.
alpha. (**HRG**-.**alpha**.), **heregulin**-.
beta.1 (**HRG**-.**beta**.1), **heregulin**-.
beta.2 (**HRG**-.**beta**.2), **heregulin**-.
beta.2-like (**HRG**-.**beta**.2-like), and
heregulin-.**beta**.3 (**HRG**-.**beta**.3).
See Holmes et al., Science 256:1205-1210 (1992); WO 92/20798; and U.S. Pat. No. 5,367,060. The 45 kDa polypeptide, **HRG**-.**alpha**., was purified from the conditioned medium of the MDA-MB-231 human breast cancer cell line. These researchers demonstrated the ability of the purified **heregulin** polypeptides to activate tyrosine phosphorylation of the HER2 receptor in MCF7 breast tumor cells. Furthermore, the mitogenic activity of the **heregulin** polypeptides on SK-BR-3 cells (which express high levels of the HER2 receptor) was illustrated. Like other growth factors which belong. . .

DETD [0042] Falls et al., Cell, 72:801-815 (1993) describe another member of the **heregulin** family which they call acetylcholine receptor inducing activity (ARIA) polypeptide. The chicken-derived ARIA polypeptide stimulates synthesis of muscle acetylcholine receptors. See also WO 94/08007. ARIA is a .beta.-type **heregulin** and lacks the entire spacer region rich in glycosylation sites between the Ig-like domain and EGF-like domain of **HRG**.**alpha**., and **HRG**.**beta**.1-.**beta**.3.

DETD . . . proteins which they call glial growth factors (GGFs). These GGFs share the Ig-like domain and EGF-like domain with the other **heregulin** proteins described above, but also have an amino-terminal kringle domain. GGFs generally do not have the complete glycosylated spacer region. . .

DETD [0044] Ho et al. in J. Biol. Chem. 270(4):14523-14532 (1995) describe another member of the **heregulin** family called sensory and motor neuron-derived factor (SMDF). This protein has an EGF-like domain characteristic of all other **heregulin** polypeptides but a distinct N-terminal domain. The major structural difference between SMDF and the other **heregulin** polypeptides is the lack in SMDF of the Ig-like domain and the "glyco" spacer characteristic of all the other **heregulin** polypeptides. Another feature of SMDF is the presence of two stretches of hydrophobic amino acids near the N-terminus.

DETD [0045] While the **heregulin** polypeptides were first identified based on their ability to activate the HER2 receptor (see Holmes et al., supra), it was. . . undergo tyrosine phosphorylation (Peles et al., EMBO J. 12:961-971 (1993)). This indicated another cellular component was necessary for conferring full **heregulin** responsiveness. Carraway et al. subsequently demonstrated that .sup.125I-**rHRG**.beta.1.sub.177-244 bound to NIH-3T3 fibroblasts stably transfected with bovine erbB3 but not to non-transfected parental cells. Accordingly, they conclude that ErbB3. . . (1994). Sliwkowski et al., J. Biol. Chem. 269(20):14661-14665 (1994) found that cells transfected with HER3 alone show low affinities for **heregulin**, whereas cells transfected with both HER2 and HER3 show higher affinities.

DETD . . . p185.sup.HER4/p185.sup.HER2 activation. They expressed p185.sup.HER2 alone, p185.sup.HER4 alone, or the two receptors together in human T lymphocytes and demonstrated that **heregulin** is capable of stimulating tyrosine phosphorylation of p185.sup.HER4, but could only stimulate p185.sup.HER2 phosphorylation in cells expressing both receptors. Plowman. . .

DETD [0048] The biological role of **heregulin** has been investigated by several groups. For example, Falls et al., (discussed above) found

that ARIA plays a role in. . .

DETD . . . factor for astrocytes (Pinkas-Kramarski et al., PNAS, USA 91:9387-9391 (1994)). Meyer and Birchmeier, PNAS, USA 91:1064-1068 (1994) analyzed expression of **heregulin** during mouse embryogenesis and in the perinatal animal using in situ hybridization and RNase protection experiments. See also Meyer et al., Development 124(18):3575-3586 (1997). These authors conclude that, based on expression of this molecule, **heregulin** plays a role in vivo as a mesenchymal and neuronal factor. Similarly, Danilenko et al., Abstract 3101, FASEB 8(4-5):A535 (1994);. . .

DETD [0057] "**Heregulin**" ligand is defined herein to be any isolated ligand, preferably a polypeptide sequence which possesses a biological property of a naturally occurring **heregulin** polypeptide that binds and activates Her2. Ligands within the scope of this invention include the **heregulin** polypeptides discussed in detail herein. **Heregulin** includes the polypeptides shown in FIGS. 1A-1D, 2A-2E, 3A-3E, 4A-4C, 5A-5D, 6A-6C, and 7A-7C and mammalian analogues thereof. Variants can. . .

DETD [0058] The term a "normal" hair **cell** or **inner-ear**-supporting **cell** means an hair **cell** or **inner-ear**-supporting **cell** which is not transformed, i.e., is non-cancerous and/or non-immortalized. Further, the normal hair **cell** or **inner-ear**-supporting **cell** is preferably not aneuploid. Aneuploidy exists when the nucleus of a **cell** does not contain an exact multiple of the haploid number of chromosomes, one or more chromosomes being present in greater or lesser number than the rest. Typical properties of transformed **cells** which fall outside the scope of this invention include the ability to form tumors when implanted into immune-deprived mice (nude mice), the ability to **grow** in suspension or in semi-solid media such as agar, a loss of contact inhibition allowing piling up of **cells** into colonies or foci, a loss of dependence on **growth** factors or serum, **cell** death if **cells** are inhibited from **growing**, and disorganization of actin filaments. Specifically included within the invention are normal **cells** which will not form tumors in mice, **grow** attached to plastic or glass (are anchorage dependent), exhibit contact inhibition, require serum-containing hormones and **growth** factors, remain viable if **growth** is arrested by lack of serum, and contain well-organized actin filaments. Although the normal **inner-ear**-supporting **cells** are preferably not cultured **cells**, also suitable for the invention are non-transformed, non-immortalized epithelial **cells** isolated from mammalian tissue. These isolated **cells** may be cultured for several **generations** (up to about 10 or even 50 **generations**) in the presence of a **heregulin** in order to induce **growth** and/or **proliferation** of the isolated **inner ear** supporting **cell** sample, that is, to expand the sample. The expanded sample can then be reintroduced into the mammal for the purpose of repopulating the hair **cell** or **inner-ear**-supporting **cell** tissue (re-epithelialization). This is particularly useful for repairing tissue injury or damage.

DETD . . . purposes herein means an in vivo biologic or antigenic function or activity that is directly or indirectly performed by an **heregulin** sequence (whether in its native or denatured conformation), or by any subsequence thereof. Biologic functions include receptor binding, any enzyme. . . i.e. possession of an epitope or

antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring **heregulin** polypeptide.

DETD [0060] "Biologically active" **heregulin** is defined herein as a polypeptide sharing a biologic function of an **heregulin** sequence which may (but need not) in addition possess an antigenic function. A principal known effect or function of **heregulin** is as a ligand polypeptide having a qualitative biological activity of binding to HER2 resulting in the activation of the receptor tyrosine kinase (an "activating ligand"). One test for activating ligands is the **heregulin** tyrosine autophosphorylation assay described below. Included within the scope of **heregulin** as that term is used herein are **heregulin** having translated mature amino acid sequences of the complete human **heregulin** as set forth herein; deglycosylated or unglycosylated derivatives of **heregulin**, amino acid sequence variants of **heregulin** sequence, and derivatives of **heregulin**, which are capable of exhibiting a biological property in common with **heregulin**. While native **heregulin** is a membrane-bound polypeptide, soluble forms, such as those forms lacking a functional transmembrane domain, are also included within this definition. In particular, included are polypeptide fragments of **heregulin** sequence which have an N-terminus at any residue from about S216 to about A227, and its C-terminus at any residue. . .

DETD [0061] "Antigenically active" **heregulin** is defined as a polypeptide that possesses an antigenic function of an **heregulin** and which may (but need not) in addition possess a biologic function.

DETD [0062] In preferred embodiments, antigenically active **heregulin** is a polypeptide that binds with an affinity of at least about 10.sup.-9 I/mole to an antibody raised against a naturally occurring **heregulin** sequence. Ordinarily the polypeptide binds with an affinity of at least about 10.sup.-8 I/mole. Most preferably, the antigenically active **heregulin** is a polypeptide that binds to an antibody raised against one of heregulins in its native conformation. **Heregulin** in its native conformation generally is **heregulin** as found in nature which has not been denatured by chaotropic agents, heat or other treatment that substantially modifies the three dimensional structure of **heregulin** as determined, for example, by migration on nonreducing, nondenaturing sizing gels. Antibody used in this determination may be rabbit polyclonal antibody raised by formulating native **heregulin** from a non-rabbit species in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of anti-**heregulin** antibody plateaus.

DETD [0063] Ordinarily, biologically or antigenically active **heregulin** will have an amino acid sequence having at least 75% amino acid sequence identity with a given **heregulin** sequence, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to an **heregulin** sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with **heregulin** residues in the **heregulin** of FIG. 6A-6C, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not. . . any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into **heregulin** sequence shall be construed as affecting homology.

DETD [0064] Thus, the biologically active and antigenically active

heregulin polypeptides that are the subject of this invention include each entire **heregulin** sequence; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues from **heregulin** sequence; amino acid sequence variants of **heregulin** sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, **heregulin** sequence or its fragment as defined above; amino acid sequence variants of **heregulin** sequence or its fragment as defined above has been substituted by another residue. **heregulin** polypeptides include those containing predetermined mutations by, e.g., site-directed or PCR mutagenesis, and other animal species of **heregulin** polypeptides such as rabbit, rat, porcine, non-human primate, equine, murine, and ovine **heregulin** and alleles or other naturally occurring variants of the foregoing and human sequences; derivatives of **heregulin** or its fragments as defined above wherein **heregulin** or its fragments have been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope); glycosylation variants of **heregulin** (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of appropriate amino acid); and soluble forms of **heregulin**, such as HRG-GFD or those that lack a functional transmembrane domain.

DETD [0065] "Isolated" means a ligand, such as **heregulin**, which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for **heregulin**, and may include proteins, hormones, and other substances. In preferred embodiments, **heregulin** will be purified (1) to greater than 95% by weight of protein as determined by the Lowry method or other. . . marketed on the filing date hereof, or (3) to homogeneity by SDS-PAGE using Coomassie blue or, preferably, silver stain. Isolated **heregulin** includes **heregulin** in situ within recombinant cells since at least one component of **heregulin** natural environment will not be present. Isolated **heregulin** includes **heregulin** from one species in a recombinant cell culture of another species since **heregulin** in such circumstances will be devoid of source polypeptides. Ordinarily, however, isolated **heregulin** will be prepared by at least one purification step.

DETD [0066] In accordance with this invention, **heregulin** nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active **heregulin**, is complementary to nucleic acid sequence encoding such **heregulin** , or hybridizes to nucleic acid sequence encoding such **heregulin** and remains stably bound to it under stringent conditions.

DETD [0067] Preferably, **heregulin** nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with an **heregulin** sequence. Preferably, the **heregulin** nucleic acid that hybridizes contains at least 20, more preferably at least about 40, and most preferably at least about. . .

DETD [0068] Isolated **heregulin** nucleic acid includes a nucleic acid that is identified and separated from at least one containment nucleic acid with which it is ordinarily associated in the natural source of **heregulin** nucleic acid. Isolated **heregulin** nucleic

acid thus is present in other than in the form or setting in which it is found in nature. However, isolated **heregulin** encoding nucleic acid includes **heregulin** nucleic acid in ordinarily **heregulin**-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature. Nucleic acid encoding **heregulin** may be used in specific hybridization assays, particularly those portions of **heregulin** encoding sequence that do not hybridize with other known DNA sequences. "Stringent conditions" are those that (1) employ low ionic. . .

DETD [0081] The "**heregulin** tyrosine autophosphorylation assay" to detect the presence or bioactivity of **heregulin** ligands can be used to monitor the purification of a ligand for the HER2 receptors. This assay is based on. . .

DETD [0097] II. Use and Preparation of **Heregulin** Sequences

DETD [0098] H. Preparation of **Heregulin** Sequences, Including Variants

DETD [0099] The system to be employed in preparing **heregulin** sequence will depend upon the particular **heregulin** sequence selected. If the sequence is sufficiently small **heregulin** may be prepared by in vitro polypeptide synthetic methods. Most commonly, however, **heregulin** will be prepared in recombinant cell culture using the host-vector systems described below. Suitable **heregulin** includes any biologically active and antigenetically active **heregulin**.

DETD . . . In general, mammalian host cells will be employed, and such hosts may or may not contain post-translational systems for processing **heregulin** preprosequences in the normal fashion. If the host cells contain such systems then it will be possible to recover natural. . . vitro method. However, it is not necessary to transform cells with the complete prepro or structural genes for a selected **heregulin** when it is desired to only produce fragments of **heregulin** sequences. For example, a start codon is ligated to the 5' end of DNA encoding an HRG-GFD, this DNA is. . .

DETD [0101] **Heregulin** sequences located between the first N-terminal mature residue and the first N-terminal residue of HRG-GFD sequence, termed HRG-NTD, may function. . . molecule but from expression of DNA in which a stop codon is located at the C-terminus of HRG-NTD. In addition, **heregulin** variants are expressed from DNA encoding protein in which both the GFD and NTD domains are in their proper orientation. . . and (2) a stop codon is introduced in the sequence RCT or RCQ in place of cysteinyl, or threonyl (for **HRG**-.**alpha**.) or glutaminyl (for **HRG**-.**beta**.).

DETD [0102] A preferred **HRG**-.**alpha**.ligand with binding affinity to p185.sup.HER2 comprises amino acids 226-265 of FIG. 1A-D. This **HRG**-.**alpha**. ligand further may comprise up to an additional 1-20 amino acids preceding amino acid 226 and 1-20 amino acids following amino acid 265. A preferred **HRG**-.**beta** . ligand with binding affinity to p185.sup.HER2 comprises amino acids 226-265 of FIG. 2A-E. This **HRG**-.**beta**. ligand may comprise up to an additional 1-20 amino acids preceding amino acid 226 and 1-20 amino acids following amino. . .

DETD [0103] As noted above, other **heregulin** sequences to be prepared in accordance with this invention are those of the GFD. These are synthesized in vitro or. . . in recombinant cell culture. These are produced most inexpensively in yeast or E.coli by secretion under the control of a **heregulin**-heterologous signal as described infra, although preparation in mammalian cells is also contemplated

using a mammalian protein signal such as that of tPA, UK or a secreted viral protein. The GFD can be the sequence of a native **heregulin** or may be a variant thereof as described below. GFD sequences include those in which one or more residues from. . .

DETD [0104] An additional **heregulin** is one which contains the GFD and the sequence between the C-terminus of GFD and the N-terminus of the transmembrane. . . C-terminal cleavage domain or CTC). In this variant (HRG-GFD-CTC) the DNA start codon is present at the 5' end of **heregulin**-heterologous signal sequence or adjacent the 5' end of the GFD-encoding region, and a stop codon is found in place of. . . about 5 residues N- and 5 residues C-terminal from this residue. It is known that Met-227 terminal and Val-229 terminal **HRG**-. **alpha**.-GFD are biologically active. The C-terminus for **HRG**-.**alpha**.-GFD may be Met-227, Lys-228, Val-229, Gln-230, Asn-231 or Gln-232, and for **HRG**-.**beta**.-GFD may be Met-226, Ala-227, Ser-228, Phe-229, Trp-230, or Lys231/Ser231. The native C-terminus is determined readily by C-terminal sequencing, although it. . .

DETD [0106] If it is desired to prepare the longer **heregulin** polypeptides and the 5' or 3' ends of the given **heregulin** are not described herein, it may be necessary to prepare nucleic acids in which the missing domains are supplied by homologous regions from more complete **heregulin** nucleic acids. Alternatively, the missing domains can be obtained by probing libraries using the DNAs disclosed in the Figures or. . .

DETD [0107] A. Isolation of DNA Encoding **Heregulin**

DETD [0108] The DNA encoding **heregulin** may be obtained from any cDNA library prepared from tissue believed to possess **heregulin** mRNA and to express it at a detectable level. **HRG**-. **alpha**. gene thus may be obtained from a genomic library. Similar procedures may be used for the isolation of other **heregulin**, such as **HRG**-.**beta**.1, **HRG**-.**beta** .2, or **HRG**-.**beta**.3 encoding genes.

DETD . . . encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind to **HRG**-.**alpha**.; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of **HRG**-.**alpha**. cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or. . .

DETD [0110] An alternative means to isolate the gene encoding **HRG**-. **alpha**. is to use polymerase chain reaction (PCR) methodology as described in section 14 of Sambrook et al., supra. This method requires the use of oligonucleotide probes that will hybridize to **HRG**-. **alpha**.. Strategies for selection of oligonucleotides are described below.

DETD . . . preferably human breast, colon, salivary gland, placental, fetal, brain, and carcinoma cell lines. Other biological sources of DNA encoding an **heregulin**-like ligand include other mammals and birds. Among the preferred mammals are members of the following orders: bovine, ovine, equine, murine,. . .

DETD . . . minimized. The actual nucleotide sequence(s) may, for example, be based on conserved or highly homologous nucleotide sequences or regions of **HRG**-.**alpha**.. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance. . .

DETD [0114] Of particular interest is **HRG**-.**alpha**. nucleic acid that encodes a full-length polypeptide. In some preferred

embodiments, the nucleic acid sequence includes the native **HRG**-.**alpha**. signal sequence. Nucleic acid having all the protein coding sequence is obtained by screening selected cDNA or genomic libraries, and,. . .

DETD [0115] **HRG**-.**alpha**. encoding DNA of FIGS. 1A-1D may be used to isolate DNA encoding the analogous ligand from other animal species via. . .

DETD [0116] The EGF-like domain fragment **HRG**-.**beta**.1 177-244 was amplified from vector pHL89 (which is described in Holmes et al., Science 256:1205-1210 (1992)) by PCR with primers. . . replacing this gene with a stuffer fragment, which provides space for cleavage at the restriction sites used for cloning. The **HRG**-.**beta**.1 fragment was attached to residue 247 of pIII.

DETD [0117] The **HRG**-.**beta**.1 EGF-like domain expressed from the above-described construct is designated by removing the "p" and the "-g3" that appear in the name of the construct. Thus, the **HRG**-.**beta**.1 EGF-like domain expressed from the pHRG2-g3 construct is designated "HRG2." The domain was displayed monovalently on phage as a pIII fusion protein, as described by Bass et al., Proteins 8:309-314 (1990). Similarly, variants **HRG**-.**beta**.1.sub.147-227, **HRG**-.**beta**.1.sub.147-244, and **HRG**-.**beta**.1.sub.177-227 were prepared and express described above.

DETD [0118] B. Amino Acid Sequence Variants of **Heregulin**

DETD [0119] Amino acid sequence variants of **heregulin** are prepared by introducing appropriate nucleotide changes into **heregulin** DNA, or by in vitro synthesis of the desired **heregulin** polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for human **heregulin** sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Excluded from the scope of this invention are **heregulin** variants or polypeptide sequences that are not novel and unobvious over the prior art. The amino acid changes also may alter post-translational processes of **HRG**-.**alpha**., such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, altering the intra-cellular location of **heregulin** by inserting, deleting, or otherwise affecting the leader sequence of the native **heregulin**, or modifying its susceptibility to proteolytic cleavage.

DETD [0120] The **heregulin** sequence may be proteolytically processed to create a number of **heregulin** fragments. HRG-GFD sequences of **HRG**-.**alpha**. all contain the amino acid sequence between **HRG**-.**alpha**. cysteine 226 and cysteine 265. The amino terminus of **HRG**-.**alpha**. fragment may result from the cleavage of any peptide bond between alanine 1 and cysteine 226, preferably adjacent to an arginine, lysine, valine, or methionine, and most preferably between methionine 45 and serine 46. The carboxy terminus of **HRG**-.**alpha**. fragment may result from the cleavage of any peptide bond between cysteine 265, preferably adjacent to an arginine, lysine, valine,. . . lysine 272 and valine 273, between lysine 278 and alanine 279, or between lysine 285 and arginine 286. The resulting **HRG**-.**alpha**. ligands resulting from such proteolytic processing are the preferred ligands.

DETD [0121] **HRG**-.**beta**.-GFD's are analogous to those discussed above for **HRG**-.**alpha**.-GFD's. Each **HRG**-.**beta**.-GFD contains the polypeptide segment from

cysteine 212 to cysteine 251 of FIG. 2A-E. The amino terminus of **HRG**-.**beta**.1 fragment may result from the cleavage of any peptide bond between alanine 1 and cysteine 212, preferably adjacent to an arginine, lysine, valine, or methionine, and most preferably between methionine 31 and serine 32. The carboxy terminus of **HRG**-.**beta**.1 fragment may result from the cleavage of any peptide bond between cysteine 251 of FIG. 2A-2E, preferably adjacent to an. . . lysine 261 and histidine 262, between lysine 276 and alanine 277, or between lysine 301 and thrionine 302. The resulting **HRG**-.**beta**.1 ligands resulting from such proteolytic processing are among the preferred ligands. Similarly, processing to produce preferred fragment ligands of **HRG**-.**beta**.2 based upon the FIG. 3A-3E and **HRG**-.**beta**.3 based upon FIG. 4A-4C may be accomplished by cleaving **heregulin** sequences of FIGS. 3A-3E and 4A-4C preferably adjacent to an arginine, lysine, valine or methionine.

DETD [0122] In designing amino acid sequence variants of **heregulin**, the location of the mutation site and the nature of the mutation will depend on **heregulin** characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first. . .

DETD [0123] A useful method for identification of certain residues or regions of **heregulin** polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244:. . . a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the expressed **heregulin** variants are screened for the optimal combination of desired activity.

DETD . . . amino acid sequence variants: the location of the mutation site and the nature of the mutation. These are variants from **heregulin** sequence, and may represent naturally occurring alleles (which will not require manipulation of **heregulin** DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon **heregulin** characteristic to be modified. Obviously, such variations that, for example, convert **heregulin** into a known receptor ligand, are not included within the scope of this invention, nor are any other **heregulin** variants or polypeptide sequences that are not novel and unobvious over the prior art.

DETD . . . contiguous. Deletions may be introduced into regions of low homology with other EGF family precursors to modify the activity of **heregulin**. Deletions from **heregulin** in areas of substantial homology with other EGF family sequences will be more likely to modify the biological activity of **heregulin** more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of **heregulin** in the affected domain, e.g., cysteine crosslinking, beta-pleated sheet or alpha helix.

DETD . . . or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within **heregulin** sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, and most preferably 1 to 3. Examples of terminal insertions include **heregulin** with an N-terminal methionyl residue (an artifact of the direct expression of **heregulin** in bacterial recombinant cell culture), and fusion of a heterologous N-terminal signal sequence to the N-terminus of **heregulin** to facilitate the secretion of mature

heregulin from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host. . .

DETD [0127] Other insertional variants of **heregulin** include the fusion to the N- or C-terminus of **heregulin** of an immunogenic polypeptide, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions of **heregulin**-ECD with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described. . .

DETD [0128] Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in **heregulin** molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of **heregulin**, and sites where the amino acids found in **heregulin** ligands from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. A likely sub-domain of HRG-GFD. . . as a growth factor is the C-terminal segment, in particular within the sequence about from glycine 218 to valine 226 (**HRG**-. **alpha**.), and glycine 218 to lysine 228/serine 228 (**HRG** -.**beta**.) based upon analogy to the EGF sub-sequence found to have EGF activity.

DETD [0129] Other sites of interest are those in which particular residues of **heregulin**-like ligands obtained from various species are identical. These positions may be important for the biological activity of **heregulin**. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a. . .

DETD [0130] Substantial modifications in function or immunological identity of **heregulin** are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone. . .

DETD . . . entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of **heregulin** that are homologous with other receptor ligands, or, more preferably, into the non-homologous regions of the molecule.

DETD [0140] Any cysteine residues not involved in maintaining the proper conformation of **heregulin** also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DETD [0141] Sites particularly suited for substitutions, deletions or insertions, or use as fragments, include, numbered from the N-terminus of **HRG**-.**alpha**. of FIG. 1A-1D:

DETD [0151] Analogous regions in **HRG**-.**beta**.1 may be determined by reference to its' sequence. The analogous **HRG**-. **beta**.1 amino acids may be mutated or modified as discussed above for **HRG**-.**alpha**.. Analogous regions in **HRG** -.**beta**.2 may also be determined by reference to its' sequence. The analogous **HRG**-.**beta**.2 amino acids may be mutated or modified as discussed above for **HRG**-.**alpha**. or **HRG**-.**beta**.1. Analogous regions in **HRG**-. **beta**.3 may be determined by reference to its' sequence. Further, the analogous **HRG**-.**beta**.3 amino acids may be mutated or modified as discussed above for **HRG**-.**alpha**., **HRG**-.**beta**.1, or **HRG**-.**beta**.2.

DETD [0152] Another **heregulin** variant is or gamma-**heregulin**

. -HRG is any polypeptide sequence that possesses at least one biological property of native sequence -HRG having SEQ ID NO:11. The biological property of this variant is the same as for **heregulin** noted above. This variant encompasses not only the polypeptide isolated from a native -HRG source such as human MDA-MB-175 cells. . . residues within the amino acid sequence shown for the human protein in FIG. 7A-7C as generally described agove for other **heregulin**. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct. . .

DETD . . . the variant has an amino acid substitution at a selected residue corresponding to a residue of 645-amino acid native human **heregulin**-.beta.1 selected from:

DETD [0156] Other **heregulin**-.beta.1 variants include an amino acid substitution selected from:

DETD [0158] In a variation of this embodiment, the **heregulin** variant includes sets of amino acid substitutions selected from this group.

DETD [0159] In addition to including one or more of the amino acid substitutions disclosed herein, the **heregulin** variant can have one or more other modifications, such as an amino acid substitution, an insertion of at least one. . . amino acid, a deletion of at least one amino acid, or a chemical modification. For example, the invention provides a **heregulin** variant that is a fragment. In a variation of this embodiment, the fragment includes residues corresponding to a portion of human **heregulin**-.beta.1 extending from about residue 175 to about residue 230 (i.e., the EGF-like domain). For example, the fragment can extend from residue 177 to residue 244 and may be prepared by recombinant techniques (**rHRG**.beta.1-177-244).

DETD [0160] DNA encoding amino acid sequence variants of **heregulin** is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation. . . by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of **heregulin**. These techniques may utilize **heregulin** nucleic acid (DNA or RNA), or nucleic acid complementary to **heregulin** nucleic acid.

DETD [0161] Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of **heregulin** DNA. This technique is well known in the art as described by Adelman et al., DNA, 2: 183 (1983). Briefly, **heregulin** DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of **heregulin**. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in **heregulin** DNA.

DETD . . . DNA encodes the mutated form of heregutin, and the other strand (the original template) encodes the native, unaltered sequence of **heregulin**. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as E. coli JM101. After. . .

DETD [0166] DNA encoding **heregulin** mutants with more than one amino acid to be substituted may be generated in one of several ways. If the. . .

DETD [0169] PCR mutagenesis is also suitable for making amino acid variants

of **heregulin**. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR. . .

DETD . . . technique described by Wells et al. (Gene, 34: 315, 1985). The starting material is the plasmid (or other vector) comprising **heregulin** DNA to be mutated. The codon(s) in **heregulin** DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified. . . restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in **heregulin** DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize. . . of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated **heregulin** DNA sequence.

DETD [0180] The cDNA or genomic DNA encoding native or variant **heregulin** is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available,. . .

DETD [0182] In general, the signal sequence may be a component of the vector, or it may be a part of **heregulin** DNA that is inserted into the vector. The native **heregulin** DNA is believed to encode a signal sequence at the amino terminus (5' end of the DNA encoding **heregulin**) of the polypeptide that is cleaved during post-translational processing of the polypeptide to form the mature **heregulin** polypeptide ligand that binds to the HER2/HER3 receptor, although a conventional signal structure is not apparent. Native **heregulin** is, secreted from the cell but remains lodged in the membrane because it contains a transmembrane domain and a cytoplasmic region in the carboxyl terminal region of the polypeptide. Thus, in a secreted, soluble version of **heregulin** the carboxyl terminal domain of the molecule, including the transmembrane domain, is ordinarily deleted. This truncated variant **heregulin** polypeptide may be secreted from the cell, provided that the DNA encoding the truncated variant encodes a signal sequence recognized. . .

DETD [0183] **Heregulin** of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a. . . polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of **heregulin** DNA that is inserted into the vector. Included within the scope of this invention are **heregulin** with the native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be. . . by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native **heregulin** signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II leaders. For yeast secretion the native **heregulin** signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the. . .

DETD . . . in Bacillus genomic DNA. Transfection of Bacillus with this vector results in homologous recombination with the genome and insertion of **heregulin** DNA. However, the recovery of genomic DNA encoding **heregulin** is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise **heregulin** DNA. DNA can be amplified by PCR and directly

transfected into the host cells without any replication component.

DETD . . . example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up **heregulin** nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which. . . in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes **heregulin**. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of **heregulin** are synthesized from the amplified DNA.

DETD . . . of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding **heregulin**. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of. . . employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding **heregulin**, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in. . .

DETD . . . Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to **heregulin** nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as **heregulin** to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters. . . promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding **heregulin** by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native **heregulin** promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of **heregulin** DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed **heregulin** as compared to the native **heregulin** promoter.

DETD . . . are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding **heregulin** (Siebenlist et al., Cell 20: 269, 1980) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding **heregulin**.

DETD [0200] **Heregulin** gene transcription from vectors in mammalian host cells may be controlled by promoters obtained from the genomes of viruses such. . . mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with **heregulin** sequence, provided such promoters are compatible with the host cell systems.

DETD [0203] Transcription of a DNA encoding **heregulin** of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting. . . for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to **heregulin** DNA, but is

preferably located at a site 5' from the promoter.

DETD . . . DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding **heregulin**. The 3' untranslated regions also include transcription termination sites.

DETD . . . the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding **heregulin**. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host. . . physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of **heregulin** that have **heregulin**-like activity. Such a transient expression system is described in U.S. Pat. No. 5,024,939.

DETD [0209] Other methods, vectors, and host cells suitable for adaptation to the synthesis of **heregulin** in recombinant vertebrate cell culture are described in Gething et al., Nature 293: 620-625, 1981; Mantei et al., Nature, 281:. . . 1979; Levinson et al., EP 117,060 and EP 117,058. A particularly useful expression plasmid for mammalian cell culture expression of **heregulin** is pRK5 (EP pub. no. 307,247) or pSVI6B (U.S. Ser. No. 07/441,574, filed Nov. 22, 1989, the disclosure of which. . .

DETD [0212] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for **heregulin** -encoding vectors. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number. . .

DETD [0213] Suitable host cells for the expression of glycosylated **heregulin** polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any. . . cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens, which has been previously manipulated to contain **heregulin** DNA. During incubation of the plant cell culture with A. tumefaciens, the DNA encoding **heregulin** is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express **heregulin** DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and. . .

DETD [0219] Prokaryotic cells used to produce **heregulin** polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

DETD [0220] The mammalian host cells used to produce **heregulin** of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal. . .

DETD [0222] It is further envisioned that **heregulin** of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding **heregulin** currently in use in the field. For example, a powerful promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element. . . genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired **heregulin**. The control element does not encode **heregulin** of this invention, but the DNA is present in the host cell genome. One next screens for cells making **heregulin** of this invention, or increased or decreased levels of expression, as desired.

DETD . . . either monoclonal or polyclonal, and may be prepared in any

mammal. Conveniently, the antibodies may be prepared against a native **heregulin** polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further below.

DETD [0227] G. Purification of The **Heregulin** Polypeptides

DETD [0228] **Heregulin** is recovered from a cellular membrane fraction. Alternatively, a proteolytically cleaved or a truncated expressed soluble **heregulin** fragment or subdomain are recovered from the culture medium as a soluble polypeptide. A **heregulin** is recovered from host cell lysates when directly expressed without a secretory signal.

DETD [0229] When **heregulin** is expressed in a recombinant cell other than one of human origin, **heregulin** is completely free of proteins or polypeptides of human origin. However, it is desirable to purify **heregulin** from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to **heregulin**. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. **Heregulin** is then be purified from both the soluble protein fraction (requiring the presence of a protease) and from the membrane fraction of the culture lysate, depending on whether **heregulin** is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation;. . .

DETD [0230] **Heregulin** variants in which residues have been deleted, inserted or substituted are recovered in the same fashion as the native **heregulin**, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a **heregulin** fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-**heregulin** column can be employed to absorb **heregulin** variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenylmethylsulfonylfluoride (PMSF) also may. . . to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native **heregulin** may require modification to account for changes in the character of **heregulin** variants or upon expression in recombinant cell culture.

DETD [0231] H. Covalent Modifications of **Heregulin**

DETD [0232] Covalent modifications of **heregulin** polypeptides are included within the scope of this invention. Both native **heregulin** and amino acid sequence variants of **heregulin** optionally are covalently modified. One type of covalent modification included within the scope of this invention is a **heregulin** polypeptide fragment. **Heregulin** fragments, such as HRG-GDF, having up to about 40 amino acid residues are conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length **heregulin** polypeptide or **heregulin** variant polypeptide. Other types of covalent modifications of **heregulin** or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of **heregulin** or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N-. . .

DETD [0239] Derivatization with bifunctional agents is useful for crosslinking **heregulin** to a water-insoluble support matrix or surface for use in a method for purifying anti-**heregulin**

antibodies, and vice versa, Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid,. . .

DETD [0242] **Heregulin** optionally is fused with a polypeptide heterologous to **heregulin**. The heterologous polypeptide optionally is an anchor sequence such as that found in a phage coat protein such as M13 gene III or gene VIII proteins. These heterologous polypeptides can be covalently coupled to **heregulin** polypeptide through side chains or through the terminal residues.

DETD [0243] **Heregulin** may also be covalently modified by altering its native glycosylation pattern. One or more carbohydrate substitutents in these embodiments, are modified by adding, removing or varying the monosaccharide components at a given site, or by modifying residues in **heregulin** as that glycosylation sites are added or deleted.

DETD [0245] Glycosylation sites are added to **heregulin** by altering its amino acid sequence to contain one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites).. . . alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to **heregulin** (for O-linked glycosylation sites). For ease, **heregulin** is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding **heregulin** at preselected bases such that codons are generated that will translate into the desired amino acids.

DETD [0246] Chemical or enzymatic coupling of glycosides to **heregulin** increases the number of carbohydrate substituents. These procedures are advantageous in that they do not require production of the polypeptide. . .

DETD [0247] Carbohydrate moieties present on an **heregulin** also are removed chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an. . . al. (Arch. Biochem. Biophys., 259:52 (1987)) and by Edge et al. (Anal. Biochem., 118:131 (1981)). Carbohydrate moieties are removed from **heregulin** by a variety of endo- and exo-glycosidases as described by Thotakura et al. (Meth. Enzymol., 138:350 (1987)).

DETD [0249] **Heregulin** may also be modified by linking **heregulin** to various nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos.. . .

DETD [0250] One preferred way to increase the in vivo circulating half life of non-membrane bound **heregulin** is to conjugate it to a polymer that confers extended half-life, such as polyethylene glycol (PEG). (Maxfield, et al, Polymer. . .

DETD [0251] **Heregulin** may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or. . .

DETD . . . in order to select the optimal variant for the purpose intended. For example, a change in the immunological character of **heregulin**, such as a change in affinity for a given antigen or for the HER2 receptor, is measured by a competitive-type immunoassay using a standard or control such as a native **heregulin** (in particular native **heregulin**-GFD). Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability. . .

DETD . . . or by FACS using celi-bound receptor and labeled candidate

antibody. Agonist antibodies are preferably antibodies which stimulate autophosphorylation in the **heregulin** tyrosine autophosphorylation assay described above.

DETD [0271] The **heregulin** are used in the present invention to induce **inner-ear**-supporting **cell proliferation** to enhance new hair **cell generation**. These effects allow treatment of disease states associated with tissue damage, for example, ototoxic injury, or acoustic assault, degenerative hearing. . .

DETD . . . The field of cochlear implantation has also provided insights into both the short- and long-term effects of cochlear fenestration on **inner ear** function. Administration of **growth** factors to the inner ears of animals is now possible with the use of implanted catheters and miniature infusion pumps. Localized application of **heregulin** to the human **inner ear** can be performed to treat **inner ear disorders** related to hair **cell** disfunction.

DETD [0273] Therapeutic formulations of **heregulin** or agonist antibody are prepared for storage by mixing the **heregulin** protein having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, supra), in. . .

DETD [0274] **Heregulin** or agonist antibody to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The **heregulin** or antibody ordinarily will be stored in lyophilized form or in solution.

DETD [0275] Therapeutic **heregulin** or antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag. . .

DETD [0276] The route of **heregulin** or antibody administration is in accord with known methods, e.g., injection or infusion administration to the inner ear, or intralesional routes, or by sustained release systems as noted below. The **heregulin** ligand may be administered continuously by infusion or by bolus injection. An agonist antibody is preferably administered in the same. . .

DETD [0277] The **heregulin**, **heregulin** variant or fragment and agonist antibodies may be spray dried or spray freeze dried using known techniques (Yeo et al,. . .

DETD [0279] Sustained-release **heregulin** or antibody compositions also include liposomally entrapped **heregulin** or antibody. Liposomes containing **heregulin** or antibody are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Nat. Acad. Sci. USA, 82:. . . which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal **heregulin** therapy. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

DETD . . . of infection of a mammal by administration of an aminoglycoside antibiotic, the improvement comprising administering a therapeutically effective amount of **heregulin** or agonist, to the patient in need of such treatment to reduce or prevent ototoxin-induced hearing impairment associated with the. . .

DETD [0282] Also provided herein are methods for promoting new **inner ear** hair **cells** by inducing **inner ear** supporting **cell proliferation**, **regeneration**, or **growth** upon, prior to, or after exposure to an agent or effect that is capable of inducing a hearing or balance impairment or **disorder**. Such agents and effects are those described herein.

The method includes the step of administering to the **inner ear** hair **cell** an effective amount of **heregulin** or agonist or factor disclosed herein as useful. Preferably, the method is used upon, prior to, or after exposure to. . .

DETD [0287] The **heregulin** or agonist is directly administered to the patient by any suitable technique, including parenterally, intranasally, intrapulmonary, orally, or by absorption. . .

DETD [0288] The **heregulin** or antibody agonist, can be combined and directly administered to the mammal by any suitable technique, including infusion and injection.. . . of administration will depend, e.g., on the medical history of the patient, including any perceived or anticipated side effects using **heregulin** alone, and the particular disorder to be corrected. Examples of parenteral administration include subcutaneous, intramuscular, intravenous, intraarterial, and intraperitoneal administration.. . .

DETD [0290] An effective amount of **heregulin** or antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Also, the amount of **heregulin** polypeptide will generally be less than the amount of an agonist antibody. Accordingly, it will be necessary for the therapist. . . 1 mg/kg and up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer **heregulin** or antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored. . .

DETD [0291] In a further embodiment, **inner-ear**-supporting **cells** may be obtained or isolated from a mammalian tissue to obtain a normal **inner-ear**-supporting **cell** sample using techniques well known in the art (biopsy, etc.). This sample may then be treated with a **heregulin** protein in order to induce hair **cell** or **inner-ear** -supporting **cell growth** and/or **proliferation** in the sample thereby expanding the population of **inner-ear**-supporting **cells**. Typically, **heregulin** will be added to the in vitro **inner-ear**-supporting **cell** culture at a concentration of about 0.1 to about 100 nM preferably 1-50 nM. If desired, the primary **inner-ear** -supporting **cells** may be cultured in vitro for several **generations** in order to sufficiently expand the hair **cell** or **inner-ear**-supporting **cell** population. The hair **cell** or **inner-ear** -supporting **cells** are cultured under conditions suitable for mammalian **cell** culture as discussed above. After expansion, the expanded sample is reintroduced into the mammal for the purpose of re-epithelializing the. . .

DETD [0292] The methods and procedures described herein with respect to **HRG-.alpha.** or **heregulin** in general may be applied similarly to other **heregulin** such as **HRG-.beta**.1, **HRG-.beta**.2 and **HRG-.beta**.3 and to variants thereof, as well as to the antibodies. All references cited in this specification are expressly incorporated by. . .

DETD [0293] Characterization of **Inner-Ear**-Supporting **Cell** Cultures

DETD [0299] A much greater number of BrdU-positive cells were seen in the cultures containing **heregulin** (**HRG-.beta** .1-177-244) than any of the other factors known to activate Her receptors. Cell counts performed from the control cultures and cultures

containing confirmed that **heregulin** significantly enhanced proliferation of the utricular supporting cells ($p<0.0001$, FIG. 9). IGF-1 at 100 nM, TGF-.alpha. at 100 nM (R. . . et al., EMBO Journal 16(6):1268-78 (1997)), and IGF1-binding protein at 100 nM were weaker mitogens, if at all, compared to **heregulin**. SMDF polypeptides are prepared as described in WO 96/15244. Neuregulin-3, a neural tissue-enriched protein that binds and activates erbB4, was. . . Sciences, 94(18):9562-7 (1997). .beta.-cellulin was prepared as described in Daly et al., Cancer Research. 57(17):3804-11 (1997). The EGF-like domain of **HRG.beta**.1.sub.(177-244) was expressed in E. coli, purified and radioiodinated as described previously (Sliwkowski et al. J. Biol. Chem. 269:14661-14665 (1994)). All. . .

DETD [0300] To determine whether the effect of **heregulin** was dose-dependent, a dose-dependent study was carried out in the utricular epithelial sheet cultures at a range of 0.03 nM to 10 nM **heregulin** (FIG. 10). A **heregulin**-dose-dependent increase in the number of BrdU positive cells was observed. Maximal effect of **heregulin** was seen at 3 nM.

DETD . . . in Zheng et al. (Journal of Neuroscience, 17(21):8270-82 (1997)). This system provides an excellent means to test the effect of **heregulin** on supporting cell proliferation in a physiologically significant system that mimics the in vivo state. In particular, the effects of **heregulin** after ototoxic-induced damage (e.g. antibiotic gentamycin) were examined.

DETD . . . mounts were cultured 1-2 days after explant, then treated with gentamycin (1 mM) for two days, and then treated with **heregulin** (3 nM) for 11 days in the presence of tritiated thymidine. To determine the number of labeled **cells**, the tissue was fixed, sectioned and processed for autoradiography. In response to **heregulin**, compared to control cultures, an increase in the number of .sup.3H-thymidine labeled **cells** in both the supporting **cell** layer (SC) and the hair **cell** layer (HC) was observed as shown in FIGS. 11A-D, which represent similarly treated samples. The **cell** count of .sup.3H-thymidine labeled **cells** in both the supporting **cell** layer and in the hair **cell** layer increased significantly compared to control cultures lacking **heregulin** as shown in FIG. 12. The data is consistent with the data obtained in the utricular sheet cultures. And the data indicates that **heregulin** can act to increase **inner-ear**-supporting **cell** **proliferation**, which leads to hair **cell** **generation**, in instances following hair **cell** damage and injury.

DETD [0306] **Heregulin** Acts through the Her2 Receptor

DETD [0307] To provide further evidence that **heregulin** is a physiologically relevant factor and that it acts through a physiologically relevant receptor, the mRNA expression levels of **heregulin** and its receptors Her2, Her3 and Her4 in the hair **cell** and supporting **cell** layers of the rat utricular sensory epithelium were determined. RNA was extracted from the P3 utricle sheet cultures and also from UEC4 **cells** (a **inner-ear**-supporting **cell** line). Using TaqMan PCR analysis with appropriate gene-specific primers (Heid et al., Genome Research. 6(10):986-94 (1996)), it was observed that all four were expressed in the **inner ear**, however, **heregulin** and Her2 were expressed at a higher level than either Her3 or Her4 (see FIG. 13). Her4 was not expressed in the **inner**

-**ear**-supporting **cell** line.

DETD . . . monoclonal antibody was used to immunostain rat P0 (day zero) cochlea and adult utricle. Her2 was localized to the hair **cell** and supporting **cell** sensory epithelium layers in the **inner ear** (see FIG. 14 A (cochlea) and FIG. 14B (utricle)). Anti-HER2 monoclonal antibodies 2C4 and 4D5 have been described elsewhere (Fendly et al. Cancer Research 50:1550-1558 (1990)). Consistent with this observation is that immunostaining with a **heregulin** antibody suggests that **heregulin** is expressed by hair **cells** of the **inner ear**.

DETD . . . Her2, but not the addition of the immunoadhesin Her4-IgG, at saturating amounts to the utricular cultures, blocked the effects of **heregulin**. Thus, **heregulin** stimulates supporting cell proliferation and hence the generation of new hair cells by activating a Her2-mediated signaling pathway, but not. . .

DETD [0310] In addition, preliminary experiments with embryonic rat **inner ear** explant cultures show that **heregulin** affects hair **cell** differentiation by enhancing **proliferation** of hair **cell** progenitors. Rat E14 otocyst cultures treated with **heregulin** respond with an increase in the number of hair **cell** progenitor **cells** compared to untreated cultures. This is consistent with the adult tissue studies, indicating that **heregulin** stimulates the **proliferation** of **cells** that differentiate into hair **cells**.

DETD [0311] **Heregulin** Acts In Vivo to Enhance **Inner Ear** Supporting **Cell Proliferation** and Hair **Cell Generation** Following Ototoxic Injury and Acoustic Assault

DETD [0312] Chinchillas are an accepted model to test the effects of factors and agents against or following hair **cell** damage or injury. Chinchillas can be treated with gentamicin, caboplatin or acoustic trauma. Preferably, at least five chinchillas are in. . . assault and allowed to recover. Typically, four to six weeks is sufficient for recovery. The test group is treated with **heregulin** in addition to the injury. All animals will receive BrdU, preferably subcutaneous infusion, using minipumps, to label the dividing **cells** during the treatment period. **Heregulin**, or one of the **heregulin** factors as taught herein, will be administered to the **inner ear**. Minipumps can be used. The **heregulin** can be infused into the cochlea. After the treatment period, cochlea and utricular maculae are dissected out of the animals. The tissue is fixed and BrdU immunohistochemical labeling done. BrdU labeled **cells** in the **inner ear** sensory epithelium are counted. **Cell** counts from the two groups--are compared and analyzed statistically to determine the amount of enhancement of **proliferation** of supporting **cells** and new hair **cell generation** induced by the **heregulin** treatment.

DETD [0532] Forge A, Li L, Corwin J T, Nevill G (1993) Ultrastructural evidence for hair **cell regeneration** in the mammalian **inner ear**. Science 259:1616-1619.

DETD [0568] Lambert P R (1994) **Inner ear** hair **cell regeneration** in a mammal: identification of a triggering factor. Laryngoscope 104:701-718.

DETD [0606] Tsue T T, Oesterle E C, Rubel E W (1994a) Diffusible factors regulate hair **cell regeneration** in the avian **inner ear**. Proc Natl Acad. Sci USA 91:1584-1588.

DETD [0607] Tsue T T, Oesterle E C, Rubel E W (1994b) Hair **cell regeneration** in the **inner ear**. Otolaryngol. Head Neck Surg 111:281-301.

DETD . . . S., Patterson, S. L., Heuer, J. G., Wheeler, E. F., Bothwell, M., and Rubel, E. W. (1991). Expression of nerve **growth** factor (NGF) receptors in the developing **inner ear** of chick and rat. Development 113: 455-470.

DETD [0612] Warchol, M. E., Lambert, P. R., Goldstein, B. J., Forge, A., and Corwin, J. T. (1993). **Regenerative proliferation** in **inner ear** sensory epithelia from adult Guinea pigs and humans. Science 259:1619-1622.

DETD [0618] Yamashita H, Oesterle E C (1995) Induction of **cell proliferation** in mammalian **inner-ear** sensory epithelia by transforing **growth** factor a and epidermal **growth** factor. Proc Natl Acad Sci USA 92:3152-3155.

CLM What is claimed is:

1. A method of inducing hair **cell generation** or **inner-ear**-supporting **cell growth**, **regeneration**, and/or **proliferation**, comprising contacting an **inner-ear**-supporting **cell** which expresses HER2 and/or HER3 receptors with an effective amount of an isolated ligand which activates HER2 and/or HER3 receptors. . .

2. The method of claim 1, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the HER2 or HER3 receptor.

3. The method of claim 2, wherein the activating ligand is human **heregulin** or a fragment thereof.

4. The method of claim 2, wherein the activating ligand is selected from the group consisting of **HRG**-.**alpha**., -.**beta** .1, -.beta.2, -.beta.2-like, and -.beta.3 and fragments thereof.

6. The method of claim 2, wherein the activating ligand is recombinant human **heregulin** or a fragment thereof.

11. The method of claim 6, wherein the **heregulin** is **rHRG**-.beta.1-177-244.

12. The method of claim 1, wherein the **inner-ear** -supporting **cell** is in the utricle or cochlea.

13. The method of claim 1 wherein the **inner-ear** -supporting **cell** expresses HER2, HER3, or both.

14. A method of increasing the number of **inner ear** supporting **cells**, comprising administering to a patient in need thereof an effective amount of an isolated HER2 and/or HER3 activating ligand.

15. The method of claim 14, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant, **heregulin** agonist antibody or fragment thereof capable of binding to the HER2 and/or HER3 receptor.

17. The method of claim 16, wherein the activating ligand is a **heregulin** polypeptide, **heregulin** variant,

heregulin agonist antibody or fragment thereof capable of binding to the HER2 and/or HER3 receptor.

18. A method, comprising the steps of: (a) obtaining an **inner-ear**-supporting **cell** sample from a mammal; (b) contacting the sample with a ligand which activates HER2 or HER3 or a combination thereof to induce **growth** and/or **proliferation** of **inner-ear**-supporting **cells** in the sample and to obtain an expanded sample; and (c) re-introducing the expanded sample into the mammal.

L7 ANSWER 2 OF 7 CAPLUS COPYRIGHT 2002 ACS

AB Immunolabeling of **heregulin**, a growth factor that enhances cell proliferation in damaged utricles, and one of its binding receptors, ErbB-2, has been briefly described in the P3 rat cochlea and utricle. However, little is known about the distribution of **heregulin** and its three binding receptors in adult animals. Here the authors describe the immunolabeling patterns for **heregulin**, ErbB-2, ErbB-3 and ErB-4 in the cochlea, spiral ganglion, utricle and saccule of the adult chinchilla using confocal microscopy. **Heregulin** immunolabeling was intense along the apical pole of Deiters cells and Hensen cells and along the membrane of supporting cells of the utricle and saccule; light immunolabeling was present in the outer layer of the spiral prominence and cytoplasm of spiral ganglion neurons. In the cochlea, intense to moderate ErbB-2 immunolabeling was evident in the cytoplasm of pillar cells, outer hair cells (OHCs), border cells, stria vascularis and spiral ligament; moderate ErbB-2 immunolabeling was present in the cytoplasm of the hair cell and supporting cell layers of the utricle and saccule. In the cochlea, light ErbB-3 immunolabeling was present in the inner hair cells, OHCs, marginal and intermediate cell layers of the stria vascularis and spiral ganglion neurons; moderate ErbB-3 immunolabeling was present in the cytoplasm of hair cells and supporting cells of the utricle and saccule. In the cochlea, utricle and saccule, ErbB-4 immunolabeling was intense in the nuclei and light to moderate in the cytoplasm and membrane of sensory cells and supporting cells. These results suggest that **heregulin** acting through ErbB receptors and various receptor complexes may play an important role in cell proliferation and survival in the cochlea and vestibular system.

ACCESSION NUMBER: 2002:529563 CAPLUS
TITLE: Expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium
AUTHOR(S): Zhang, Mei; Ding, Dalian; Salvi, Richard
CORPORATE SOURCE: Hearing Research Lab, University at Buffalo, Buffalo, NY, 14214, USA
SOURCE: Hearing Research (2002), 169(1-2), 56-68
CODEN: HERED3; ISSN: 0378-5955
PUBLISHER: Elsevier Science B.V.
DOCUMENT TYPE: Journal
LANGUAGE: English
REFERENCE COUNT: 79 THERE ARE 79 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

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However, little is known about the distribution of **heregulin** and its three binding receptors in adult animals. Here the authors describe the immunolabeling patterns for **heregulin**, ErbB-2, ErbB-3 and ErB-4 in the cochlea, spiral ganglion, utricle and saccule of the adult chinchilla using confocal microscopy. **Heregulin** immunolabeling was intense along the apical pole of Deiters cells and Hensen cells and along the membrane of supporting cells of the utricle and saccule; light immunolabeling was present in the outer layer of the spiral prominence and cytoplasm of spiral ganglion neurons. In the cochlea, intense to moderate ErbB-2 immunolabeling was evident in the cytoplasm of pillar cells, outer hair cells (OHCs), border cells, stria vascularis and spiral ligament; moderate ErbB-2 immunolabeling was present in the cytoplasm of the hair cell and supporting cell layers of the utricle and saccule. In the cochlea, light ErbB-3 immunolabeling was present in the inner hair cells, OHCs, marginal and intermediate cell layers of the stria vascularis and spiral ganglion neurons; moderate ErbB-3 immunolabeling was present in the cytoplasm of hair cells and supporting cells of the utricle and saccule. In the cochlea, utricle and saccule, ErbB-4 immunolabeling was intense in the nuclei and light to moderate in the cytoplasm and membrane of sensory cells and supporting cells. These results suggest that **heregulin** acting through ErbB receptors and various receptor complexes may play an important role in cell proliferation and survival in the cochlea and vestibular system.

ST **heregulin** ErbB receptor ear cochlea vestibule sensory epithelium

IT Ear
(cochlea; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

IT Growth factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(erbB-3; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

IT Cell membrane
Cell nucleus
Chinchilla
Cytoplasm
(expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

IT Heregulins
neu (receptor)
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

IT Growth factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-4; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

IT Ear
(organ of Corti, hair cell; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

IT **Ear**
(organ of Corti, **inner** hair **cell**; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

IT Ear
(organ of Corti, outer hair cell; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory

epithelium)
IT Ear
(sacculus; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)
IT Ear
(spiral ligament; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)
IT Ganglion
(spiral; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)
IT Ear
(stria vascularis; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)
IT Ear
(utricle; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)
IT Ear
(vestibule; expression of **heregulin** and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium)

L7 ANSWER 3 OF 7 CAPLUS COPYRIGHT 2002 ACS DUPLICATE 1
AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. The peptide of the invention is SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCQNYVMAS. A deriv. of the peptide with polyethylene glycol, dextran or a polyamino acid can also be used. The peptides are expected to be useful to treat vestibular **disorders** such as, for example, loss of balance, and to treat hearing loss.
ACCESSION NUMBER: 2000:67485 CAPLUS
DOCUMENT NUMBER: 132:88182
TITLE: Use of NDF peptide as **growth** factor for sensory epithelium of the **inner ear**
INVENTOR(S): Carnahan, Josette F.
PATENT ASSIGNEE(S): Amgen Inc., USA
SOURCE: U.S., 11 pp., Cont. of U.S. Ser. No. 129,549, abandoned.
CODEN: USXXAM
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 2
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 6017886	A	20000125	US 1999-255974	19990223

PRIORITY APPLN. INFO.: US 1998-129549 B1 19980805
REFERENCE COUNT: 17 THERE ARE 17 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT
TI Use of NDF peptide as **growth** factor for sensory epithelium of the **inner ear**
AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. The peptide of the invention is

SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCQNYVMAS. A deriv. of the peptide with polyethylene glycol, dextran or a polyamino acid can also be used. The peptides are expected to be useful to treat vestibular **disorders** such as, for example, loss of balance, and to treat hearing loss.

ST NDF peptide **growth** factor sensory epithelium **inner ear**

IT **Ear**
(**inner**; use of NDF peptide as **growth** factor for sensory epithelium for treatment of vestibular **disorders** and hearing loss)

L7 ANSWER 4 OF 7 CAPLUS COPYRIGHT 2002 ACS

AB Ligands which bind to the HER2 and/or HER3 receptors are useful as **inner-ear**-supporting **cell-growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**, e.g. in treatment of hearing **disorders**. Thus, in cultures of rat utricular epithelial sheets, **heregulin HRG-.beta.**1-177-244 significantly enhanced **proliferation** of utricular supporting **cells**. In chinchillas, **heregulin** acts in vivo to enhance **inner ear** supporting **cell proliferation** and hair **cell generation** following ototoxic injury and acoustic assault. Heregulins may also be used ex vivo for expansion of supporting **cells**, followed by reimplantation into the **inner ear**.

ACCESSION NUMBER: 2000:335267 CAPLUS
DOCUMENT NUMBER: 133:814
TITLE: Method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors
INVENTOR(S): Gao, Wei-qiang
PATENT ASSIGNEE(S): Genentech, Inc., USA
SOURCE: PCT Int. Appl., 141 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
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PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000027426	A1	20000518	WO 1999-US25744	19991028
W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
CA 2345899	AA	20000518	CA 1999-2345899	19991028
EP 1126873	A1	20010829	EP 1999-956853	19991028
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO				
US 2002081299	A1	20020627	US 2001-849868	20010504

PRIORITY APPLN. INFO.: US 1998-107522P P 19981107

WO 1999-US25744 W 19991028

REFERENCE COUNT: 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI Method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors

AB Ligands which bind to the HER2 and/or HER3 receptors are useful as **inner-ear**-supporting **cell-growth** factors to enhance **proliferation**-mediated **generation** of new hair **cells**, e.g. in treatment of hearing **disorders**. Thus, in cultures of rat utricular epithelial sheets, **heregulin HRG-.beta.**1-177-244 significantly enhanced **proliferation** of utricular supporting **cells**. In chinchillas, **heregulin** acts in vivo to enhance **inner ear** supporting **cell proliferation** and hair **cell generation** following ototoxic injury and acoustic assault. Heregulins may also be used ex vivo for expansion of supporting **cells**, followed by reimplantation into the **inner ear**.

ST ear hair **cell regeneration heregulin**; HER receptor ligand **inner ear**; **cell proliferation inner ear heregulin**

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**HRG-.alpha.**; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**HRG-.beta.**1; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**HRG-.beta.**2-like; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**HRG-.beta.**2; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**HRG-.beta.**3; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Ear

(cochlea, implant; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Ear**
(disease; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Animal tissue culture
Molecular cloning
(enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Growth** factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-3, ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Growth** factor receptors
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(**heregulin**, erbB-3, ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Antibodies
RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(**heregulin**-agonistic; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Drug delivery systems
(implants, cochlear; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Ear**
(**inner**, supporting **cell**; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT **Ear**
(**inner**, utricle; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT neu (receptor)
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(ligands; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Ear
(organ of Corti, hair **cell**; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT Heregulins

RL: BAC (Biological activity or effector, except adverse); BPR (Biological process); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); PROC (Process); USES (Uses)
(.gamma.-HRG; enhancing **proliferation** of **inner ear** hair **cells** with ligands for HER2 and/or HER3 receptors)

IT 142158-51-8 142158-52-9 142158-53-0 146591-70-0 146591-82-4 178862-39-0 196678-45-2 270245-15-3 270245-16-4, 14: PN: WO0027426 SEQID: 2 unclaimed DNA 270245-18-6
RL: PRP (Properties)
(unclaimed nucleotide sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors)

IT 146591-75-5, **Heregulin** .beta.2 (human clone .lambda.her76 precursor reduced) 146591-80-2, Protein (human clone .lambda.her84 **heregulin** .beta.2-like precursor reduced) 168183-94-6 198086-50-9, **Heregulin** (human gene .gamma.-HRG) 270245-14-2 270245-17-5
RL: PRP (Properties)
(unclaimed protein sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors)

IT 146591-69-7, 1-625-**Heregulin** .alpha. (human clone .lambda.gt10her16 precursor reduced) 146591-71-1 146591-78-8, **Heregulin** .beta.3 (human clone .lambda.her78 precursor reduced) 260348-98-9 270560-38-8 270560-39-9 270560-40-2
RL: PRP (Properties)
(unclaimed sequence; method for enhancing **proliferation** of **inner ear** hair **cells** using ligands for HER2 and/or HER3 receptors)

L7 ANSWER 5 OF 7 USPATFULL

AB Compositions, methods, and devices are provided for inducing or enhancing the **growth**, **proliferation**, **regeneration** of **inner ear** tissue, particularly **inner ear** hair **cells**. In addition, provided are compositions and methods for prophylactic or therapeutic treatment of a mammal afflicted with an **inner ear disorder** or condition, particularly for hearing impairments involving hair **cell** damage, loss, or degeneration, by administration of a therapeutically effective amount of IGF-1 or FGF-2, or their agonists, alone or in combination.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

ACCESSION NUMBER: 2000:164484 USPATFULL
TITLE: Treatment of **inner ear** hair **cells**
INVENTOR(S): Gao, Wei-Qiang, Foster City, CA, United States
PATENT ASSIGNEE(S): Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6156728		20001205
APPLICATION INFO.:	US 1997-963596		19971031 (8)

	NUMBER	DATE

09/849,868

PRIORITY INFORMATION: US 1996-29536P 19961101 (60)
US 1996-30278P 19961104 (60)
DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Moezie, F. T.
LEGAL REPRESENTATIVE: Knobbe Martens Olson & Bear, LLP.
NUMBER OF CLAIMS: 11
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 17 Drawing Figure(s); 7 Drawing Page(s)
LINE COUNT: 2344
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI Treatment of **inner ear** hair **cells**

AB Compositions, methods, and devices are provided for inducing or enhancing the **growth**, **proliferation**, **regeneration** of **inner ear** tissue, particularly **inner ear** hair **cells**. In addition, provided are compositions and methods for prophylactic or therapeutic treatment of a mammal afflicted with an **inner ear disorder** or condition, particularly for hearing impairments involving hair **cell** damage, loss, or degeneration, by administration of a therapeutically effective amount of IGF-1 or FGF-2, or their agonists, alone or. . .

SUMM This application relates to inducing, promoting, or enhancingthe **growth**, **proliferation**, or **regeneration** of **inner ear** tissue, particularly **inner ear** epithelial hair **cells**. In addition, this application provides methods, compositions and devices for prophylactic and therapeutic treatment of **inner ear disorders** and conditions, particularly hearing impairments. The methods comprise administration of insulin-like **growth** factor-I (IGF-1) and/or fibroblast **growth** factor-2 (FGF-2), or their agonists.

SUMM . . . a wide variety of causes, including infections, mechanical injury, loud sounds, aging, and chemical-induced ototoxicity that damages neurons and/or hair **cells** of the peripheral auditory system. The peripheral auditory system consistsof auditoryreceptors,hair **cells** in the organ of Corti, and primary auditory neurons, the spiral ganglion neurons in the cochlea. Spiral ganglion neurons ("SGN") are primary afferent auditory neurons that deliver signals from the peripheral auditory receptors, the hair **cells** in the organ of Corti, to the brain through the cochlear nerve. The eighth nerve connects the primary auditory neurons. . . are primary afferent sensory neurons responsible for balance and which deliver signals from the utricle, saccule and ampullae of the **inner ear** to the brain, to the brainstem. Destruction of primary afferent neurons in the spiral ganglia and hair **cells** has been attributed as a major cause of hearing impairments Damage to the peripheral auditory system is responsible for a. . .

SUMM . . . to the central nervous system may result in hearing loss. Auditory apparatus can be divided into the external and middle **ear**, **inner ear** and auditory nerve and central auditory pathways. While having some variations from species to species, the general characterization is common. . . for all mammals. Auditory stimuli are mechanically transmitted through the external auditory canal, tympanic membrane, and ossicular chain to the **inner ear**. The middle **ear** and mastoid process are normally filled with air. **Disorders** of the external and middle ear usually produce a conductive hearing loss by

interfering with this mechanical transmission. Common causes. . . space. Auditory information is transduced from a mechanical signal to a neurally conducted electrical impulse by the action of neuro-epithelial **cells** (hair **cells**) and SGN in the **inner ear**. All central fibers of SGN form synapses in the cochlear nucleus of the pontine brain stem. The auditory projections from. . . to the auditory brain stem and the auditory cortex. All auditory information is transduced by a limited number of hair **cells**, which are the sensory receptors of the **inner ear**, of which the so-called inner hair **cells**, numbering a comparative few, are critically important, since they form synapses with approximately 90 percent of the primary auditory neurons.. . . nucleus, the number of neural elements involved is measured in the hundreds of thousands. Thus, damage to a relatively few **cells** in the auditory periphery can lead to substantial hearing loss. Hence, many causes of sensorineural loss can be ascribed to lesions in the **inner ear**. This hearing loss can be progressive. In addition, the hearing becomes significantly less acute because of changes in the anatomy. . .

SUMM The toxic effects of these drugs on auditory **cells** and spiral ganglion neurons are often the limiting factor for their therapeutic usefulness. For example, antibacterial aminoglycosides such as gentamicins,. . . infection has reached advanced stages, but at this point permanent damage may already have been done to the middle and **inner ear** structure. Clearly, ototoxicity is a dose-limiting side-effect ofantibiotic administration. For example, nearly 75% of patients given 2 grams of streptomycin. . .

SUMM Accordingly, there exists a need for means to prevent, reduce or treat the incidence and/or severity of **inner ear disorders** and hearing impairments involving **inner ear** tissue, particularly **inner ear** hair **cells**, and optionally, the associated auditory nerves. Of particular interest are those conditions arising as an unwanted side-effect of ototoxic therapeutic. . . a method that provides a safe, effective, and prolonged means for prophylactic or curative treatment of hearing impairments related to **inner ear** tissue damage, loss, or degeneration, particularly ototoxin-induced and particularly involving **inner ear** hair **cells**. The present invention provides compositions and methods to achieve these goals and others as well.

SUMM The present invention is based in part on the discovery disclosed herein that the **inner ear** hair **cells** produced FGF-2 in vivo, that utricular epithelial **cells** expressed FGF receptor in vitro, and that administration of certain **growth** factors can stimulatethe production of new inner hair **cells** by inducing **proliferationof** supporting **cells** which are the hair **cell** progenitors. Among 30 **growth** factors examined, FGF-2 was the most potent mitogen. IGF-1 was also effective. Accordingly, it is an object of the invention to provide a means of inducing, promoting, or enhancing the **growth**, **proliferation**, or **regeneration** of **inner ear** tissue, particularly **inner ear** epithelial hair **cells**, in vitro, ex vivo or in vitro. It is a further object of the invention to provide a method for treating a mammal to prevent, reduce, or treat the incidence of or severity of an **inner ear** hair **cell**-related hearing impairment or **disorder** (or balance impairment), particularly an ototoxin-induced or -inducible hearing impairment, by administering

to a mammal in need of such treatment a prophylactically or therapeutically effective amount of FGF-2, IGF-1, their agonists, a functional fragment or derivative thereof, a chimeric **growth** factor comprising FGF-2 or IGF-1, a small molecule or antibody agonist thereof, or a combination of the foregoing. Optionally, a. . . a suitable interval(s) either prior to, subsequent to, or substantially concurrently with the administration of or exposure to hearing-impairment inducing **inner ear** tissue damage, preferably ototoxin-induced or -inducible hearing impairment.

DETD . . . refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) **inner ear** tissue-damage-related hearing **disorder** or impairment (or balance impairment), preferably ototoxin-induced or inducible, and involving **inner ear** hair **cells**. Those in need of treatment include those already experiencing a hearing impairment, those prone to having the impairment, and those in which the impairments are to be prevented. The hearing impairments are due to **inner ear** hair **cell** damage or loss, wherein the damage or loss is caused by infections, mechanical injury, loud sounds, aging, or, preferably, chemical-induced. . .

DETD . . . in turn impairs hearing (and/or balance). In the context of the present invention, ototoxicity includes a deleterious effect on the **inner ear** hair **cells**. Ototoxic agents that cause hearing impairments include, but are not limited to, neoplastic agents such as vincristine, vinblastine, cisplatin, taxol,. . .

DETD The patients targeted for treatment by the current invention include those patients with **inner ear** hair **cell** related conditions as defined herein.

DETD Hearing impairments relevant to the invention are preferably sensory hearing loss due to end-organ lesions involving **inner ear** hair **cells**, e.g., acoustic trauma, viral endolymphatic labyrinthitis, Meniere's disease. Hearing impairments include tinnitus, which is a perception of sound in the. . . and adenoviruses. The hearing loss can be congenital, such as that caused by rubella, anoxia during birth, bleeding into the **inner ear** due to trauma during delivery, ototoxic drugs administered to the mother, erythroblastosis fetalis, and hereditary conditions including Waardenburg's syndrome and. . . syndrome. The hearing loss can be noise-induced, generally due to a noise greater than 85 decibels (db) that damages the **inner ear**. Hearing loss includes presbycusis, which is a sensorineural hearing loss occurring as a normal part of aging, fractures of the. . . rupturing the tympanic membrane and possibly the ossicularchain, fractures affectingthe cochlea, and acoustic neurinoma, which are tumors generally of Schwann **cell** origin that arise from either the auditory or vestibular divisions of the 8th nerve. Preferably, the hearing loss is caused by an ototoxic drug that effects the auditory portion of the **inner ear**, particularly **inner ear** hair **cells**. Incorporated herein by reference are Chapters 196, 197, 198 and 199 of The Merck Manual of Diagnosis and Therapy, 14th. . .

DETD Studies in lower vertebrates and avian systems indicate that supporting **cells** in the inner ears are hair **cell** progenitors (see for example, 27 and 49). In response to injury supporting **cells** are induced to **proliferate** and differentiate into new hair **cells**. However, in the mammalian system, supporting **cell proliferation** and hair **cell regenerating** occurs at a much lower frequency than in the avian system (48, 92, 127).

The mammalian utricular epithelial supporting **cells** express epithelial antigens, including the tightjunction protein (ZO1), cytokeratin, and F-actin, but not fibroblast antigens, vimentin and Thy1.1 or glial **cell** and neuronal antigens. Characteristically,in culture, supporting **cells** require **cell**-to-**cell** contact for survival, which can be provided by other supporting **cells**, and by a fibroblast monolayer as observed with dissociated chick cochlear epithelial **cells** (16). Identification of the molecular and cellular mechanisms underlying the development and **regeneration** ofhair **cells**, has been hampered by the small tissue size, the complicated bony structures of the **inner ear**, and by the lack of hair **cell** progenitor culture systems.

DETD . . . a mammal prophylactically to prevent or reduce the occurrence or severity of a hearing (or balance) impairmentthat would result from **inner ear cell** injury, loss, or degeneration, preferably caused by an ototoxic agent, wherein a therapeutically effective amount of a **inner ear** supporting **cell growth** factor or agonist of the invention, which are compounds that promote hair **cell regeneration**, **growth**, **proliferation**, or prevent or reduce cytotoxicity of hair **cells** by induction of the **proliferation** of supporting epithelial **cells** leading to **generation** of new hair **cells**. Such molecules are agonists of the utricular epithelial **cell** FGF- and IGF-1-high-affinity binding receptors that were identified herein as expressed on the surface of sensory epithelium **cells**. Preferred compounds are FGF-2, IGF-1, agonists thereof, a functional fragment or derivative thereof, a chimeric **growth** factor comprising FGF-2 or IGF-1, such as those containing the receptor-binding sequences from FGF-2 or IGF-1, a small molecule mimic. . . or a combination of the foregoing. Optionally, a trkB or trkC agonist is also administered to the mammal when neuronal **cell** damage is also suspectedor expected. Preferablythe trkB or trkC agonist is a neurotrophin, more preferably neurotrophin NT-4/5, NT-3, or BDNF,. . . at least 80% of the binding of the natural neurotrophin ligand to the receptor. When the patient is human, the **growth** factors and neurotrophins are preferably human **growth** factors and neurotrophins or derived from human gene sequences, in part to avoid or minimize recognition of the agonist as. . .

DETD Also provided herein are methods for promoting new **inner ear** hair **cells** by inducing **inner ear** supporting **cell proliferation regeneration** , or **growth** upon, prior to, or after exposure to an agent or effect that is capable of inducing a hearing or balance impairment or **disorder**. Such agents and effects are those described herein. The method includes the step of administering to the **inner ear** hair **cell** an effective amount of FGF-2, IGF-1, or agonist thereof, or or factor disclosed herein as useful. Preferably, the method is. . .

DETD . . . of each component for purposes herein are thus determined by such considerations and are amounts that preventdamage or degeneration of **inner ear cell** function or restore **inner ear cell** function.

DETD . . . or infusions. As with the FGF-2, the IGF-I may be formulated so as to have a continual presence in the **inner ear** during the course of treatment, as described above for FGF-2. Thus, it may be covalently attached to a polymer, made into a sustained-release

formulation, or provided by implanted **cells** producing the factor.

DETD Delivery oftherapeutic agents to the **inner ear** of a subject can be done by contact with the **inner ear** or through the external auditory canal and middle ear, as by injection or via catheters, or as exemplified in U.S. Pat. No. 5,476,446, which provides a multi-functional apparatus specifically designed for use in treating and/or diagnosing the **inner ear** of a human subject. The apparatus, which is useful in the practice of the present invention, has numerous functional capabilities including but not limited to (1) deliveringtherapeutic agents into the **inner ear** or to middle-**inner ear** interface tissues; (2) withdrawing fluid materials from the **inner ear**; (3) causing temperature, pressure and volumetric changes in the fluids/fluid chambers of the **inner ear**; and (4) enabling **inner ear** structures to be electrophysiologically monitored. In addition, other systems may be used to deliver the factros and formulations of the. . . Calif. (USA). U.S. Pat. No. 4,892,538, provides an implantation device for delivery of the factors and formulations of the invention. **Cells** genetically engineered to express FGF-2, or IGF-1, or their combination, and optionally, enhancing or augmenting factors or therapeutics (e.g., trkB or trkC agonist), can be implanted in the host to provide effective levels of factor or factors. The **cells** can be prepared, encapsulated, and implanted as provided in U.S. Pat. Nos. 4,892,538, and 5,011,472, WO 92/19195, WO 95/05452, or. . .

DETD . . . a solution that is isotonic with the blood of the recipient, and even more preferably formulated for local administrationto the **inner ear**. Examples of carrier vehicles include water, saline, Ringer's solution, a buffered solution, and dextrose solution. Non-aqueous vehicles such as fixed. . . amounts of additives such as substances that enhance isotonicity and chemical stability, and when locally administered are non-toxic to the **cells** and structures of the **ear**, particularly the **inner ear**. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate,. . .

DETD . . . embodiment, agonist compositions of the invention are used during clinical organ implants or transplants to keep or improve viability of **inner ear** hair **cells**. Preferably a combination of a factors will be used as taught herein, including a trkB and a trkC agonist, with. . .

DETD . . . the Examples section herein, intact utricular epithelial sheets separated using a combined enzymatic and mechanical method essentially contain only supporting **cells** and hair **cells** (Corwin et al., 1995). The epithelial identity of the cultured **cells** was confirmed using various specific **cell** markers. While these **cells** expressed epithelial antigens including the tight junction protein (ZO1), cytokeratin and F-actin, they did not express fibroblast antigens, vimentin and Thy1.1, or glial and neuronal antigens. Most of the hair **cells** (stereocilliary bundle-bearing **cells**) were injured and many of them were dead after 2 days in culture due to their sensitivity to enzymatic digestion and mechanical trituration. Therefore, these cultures essentially represented a population of utricular supporting **cells** which are the progenitors for hair **cells** (Corwin and Cotanche, 1988; Balak et al., 1990; Rapheal, 1992; Weisleder and Rubel, 1992). These cultures provide an in vitro system to study **proliferation** and differentiation of the

inner ear supporting **cells**.

DETD The cultured **inner ear** epithelial **cells** required **cell-cell** contacts with neighboring epithelial **cells** to survive and **proliferate**. Initial attempts to culture completely-dissociatedepithelial **cells** led to virtually all **cells** dying. A requirement ofcell-cellcontact for the survival and **proliferation** of epithelial progenitors is not unprecedented and has been observed previously with brain germinal zone progenitor **cells** (Gao et al., 1991) and E9 rat neuroepithelial **cells** (Li et al., 1996). The fact that **proliferation** of neuroepithelial **cells** only occurs within the highly compact CNS ventricular zone in vivo, and in the progenitor reaggregates (Gao et al., 1991) or neurospheres (Reynolds and Weiss, 1992) in vitro, suggests the existence of a membrane-bound factor for the **growth** of neuroepithelialcells. Consistent with this idea, membrane-bound components from a C6 glioma **cell** line have been shown to be necessary for the **proliferation** and survival of dissociated, single cortical progenitorcells (Davis and Temple, 1994). In contrast to the organ culture (Warchol and Corwin, 1993). the partially dissociated epithelial **cells** grew poorly in serum-free medium, suggesting that in addition to the membrane bound molecules, soluble factors in the serum also promote the **growth** of these **cells**. A monolayerof fibroblast **cells** was reported as sufficient to support the **growth** of completely-dissociated chick cochlear epithelial **cells** (Finley and Corwin, 1995).

DETD The pure epithelial **cell** culture, along with the tritiated thymidine assay, was a rapid and convenient method to evaluateeffects of **growth** factors on **proliferation** of the **inner ear** epithelial progenitorcells. A large panel of agents could be and were examined in a relatively short time. The results of. . . data. In the present experiments, several FGF family members, namely IGF-1, IGF-2, TGF-.alpha. and EGF, were mitogenic factors for the **proliferation** of utricular supporting **cells**, from among 30 **growth** factors.

DETD . . . (1995) in the intact organ culture. One possibility for the discrepancy between these results is that the deprivation of hair **cells** in the present dissociated utricular epithelial **cell** cultures might trigger the upregulation of FGF and IGF-1 receptors and enhance the response to FGFs and IGF-1. If so, this likely reflects the situation occurring during **inner ear** injury or assault. Recently, Lee and Cotanche (1996) reported that damaging chicken cochlear epithelium by noise results in an upregulation of mRNA for the FGF receptor in the supporting **cells**. Finley and Corwin (1995) reported that FGF-2 promotes the **proliferation** of chick cochlear supporting **cells** which were completely dissociated and plated on a monolayer of fibroblast **cells**. The presence of high levels of FGF receptor and IGF-1 receptor in the **inner ear** epithelial **cells** after deprivation of hair **cells** and the inhibition of **cell proliferation** by neutralizing antibodiesaagainsteither FGF-2 or IGF-1 support the idea that FGF-2 and IGF-1 act directly on the **inner ear** supporting **cells** and induce their **proliferation** following the removal of hair **cells**. FGF-2 and IGF-1 may be candidate molecules regulating **proliferation** of the **inner ear** supporting **cells**, particularly during hair **cell regeneration** following challenge by aminoglycosides or noise.

DETD Alternatively, there may be a developmental response change to **growth** factors including FGF-2 and IGF-1 during maturation of the **inner ear** epithelium. It is possible that the mature **inner ear** epithelium responds differently relative to the developing epithelium. Exogenously added FGF-2 or IGF-1 might not elicit a **proliferation** in the intact, mature utricles (Yamashita and Oesterle, 1995) or in chick tissues which are treated with a very low. . . al., 1996) as they would in the immature utricles. Upon intensive damage by noise or drugs (massive degeneration of hair **cells**), the immature epithelium might be triggered to go back to an earlier developmental stage. Such injury induced status shift has been noticed for developing neurons (Gao and Macagno, 1988). The present study is performed on postnatal rat **inner ear cells** which are still undergoing maturation, but nonetheless is believed probativeto the influenceof FGF-2 and IGF-1 on hair **cell regeneration** after acoustic trauma or exposure to high doses of aminoglycosides in adult mammals.

DETD The finding that utricular epithelial **cells** express FGF-2 and its receptor indicates that FGF-2 is a physiological **growth** factor for the development, maintenance and/or **regeneration** of hair **cells**. FGF-2 may exert its action through an autocrine mechanism. In this model, FGF-2 produced from hair **cells** may provide their own trophic support. Recent studies have suggested that **cell** differentiation and survival in the nervous system can be regulated by a **growth** factor-mediated autocrine interaction. For instance, colocalization of neurotrophins and their mRNAs is found in developing rat forebrain (Miranda et al., 1993) and a BDNF autocrine loop regulatesthe survival of cultured dorsal root ganglion **cells** (Acheson et al., 1995). Low et al. (1995) suggested that FGF-2 protects postnatal rat cochlear hair **cells** from aminoglycoside induced injury. Alternatively, a paracrine action might also be postulated in which FGF-2 synthesized by hair **cells** could locally influence maintenance of neighboring hair **cells** and **proliferation** of supporting **cells**. In this case, degeneration of hair **cells** may lead to a burst release of FGF-2, which would in turn stimulate supporting **cell proliferationin** the **inner ear** epithelium. The latter hypothesis may explain the supporting **cell proliferation** following hair **cell** death due to acoustic trauma or exposure to aminoglycosides, since FGF-2 does not have a signal sequence and **cell** injury is a major way for its release. The data herein that anti-FGF-2 antibody, but not anti-TGF-.alpha. antibody, significantly inhibits **cell proliferation** (FIG. 7) supports this hypothesis to a certain extent. The partial, but notcomplete, blocking effect by anti-FGF-2 antibody could be attributable to possible existence of other mitogens in the culture, loss of FGF-2 (due to hair **cell** injury) during the dissociation process and/or relief from contact inhibition within the epithelium following dissociation.

DETD Similar to neurotrophins, many other **growth** factors examined in the present experiments do not show significant mitogenic effects on utricular supporting **cells**. They could, however, still be involved in later phases of hair **cell regeneration**. For example, retinoic acid can induce formation of supernumerary hair **cells** in the developing cochlea without involvement of **cell proliferation** (Kelley et al., 1993). On the other hand, early differentiating factors might inhibit the progenitor **proliferation** and push the progenitors to come out the

cell cycle and become postmitotic **cells**. Regarding this aspect, it is interesting to note then that TGF-.beta.1, TGF-.beta.2, TGF-.beta.3 and TGF-.beta.5 exhibit an inhibition on the **proliferation** of the **inner ear** epithelial **cells**. Whether such observation implies a possible involvement of TGF-.beta.s in the differentiation of hair **cells** remains to be determined.

DETD In summary, we have established a purified mammalian utricular epithelial **cell** culture, which allowed rapid examination of effects of **growth** factors on supporting **cell proliferation**, an early phase during normal development and **regeneration** of new hair **cells**. Among the 30 **growth** factors we examined, FGF-2 is the most potent mitogen. The observation that the **inner ear** hair **cells** produced FGF-2 in vivo and utricular epithelial **cells** expressed FGF receptor in vitro suggest a physiological role of FGF-2 in hair **cell** development, maintenance or **regeneration**.

DETD . . . recombinant neurotrophins (Genentech), TGF-.beta.1 (Genentech), TGF-.beta.2, TGF-.beta.3, TGF-.beta.5 (R & D Systems), activin, inhibin, glial cell derived neurotrophic factor (GDNF), **heregulin**, Gas-6, vascular endothelial growth factor (VEGF), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1, c-kit ligand (Genentech), platelet-derived growth. . .

DETD . . . growth factors have been reported to influence cell proliferationand differentiation. These include neurotrophins,the TGF-.beta. superfamily, glial cell mitogens such as **heregulin** and Gas-6, endothelial cell mitogen such as VEGF, and others listed in Table 3. When examined in these cultures, none. . .

DETD . . . 126

TGF-.beta.5 807 .+-. 59
Activin 2383 .+-. 186
Inhibin 1959 .+-. 183
GDNF 2383 .+-. 186
Schwann cell mitogens
Heregulin 2854 .+-. 179
Gas-6 2588 .+-. 95
Endothelial cell mitogen
VEGF 2156 .+-. 211
PDGF 2387 .+-. 299
CNTF 2918. . .

CLM What is claimed is:

1. A method for increasing the number of mammalian **inner ear** hair **cells**, comprising contacting mammalian **inner ear** supporting **cells** with an amount of FGF-2 that promotes **proliferation** of said **inner ear** supporting **cells**.

2. The method of claim 1 further comprising contacting said **inner ear** supporting **cells** with a supporting **cell proliferation**-inducing amount of TGF-.alpha. or a TGF-.alpha.-receptor agonist.

5. The method of claim 1, further comprising contacting said **inner ear** supporting **cells** with IGF-1 or an IGF-1 receptor agonist.

10. A method for treating a mammalian **inner ear** hair

cell related **disorder** in a mammal comprising administering to the mammal an effective amount of FGF-2 that promotes **proliferation** of **inner ear** supporting **cells**.

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AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. A monoclonal antibody against adult rat utricular epithelium is also described.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

ACCESSION NUMBER:	2000:80853 USPATFULL
TITLE:	Monoclonal antibody against utricular epithelium
INVENTOR(S):	Carnahan, Josette F., Newbury Park, CA, United States
PATENT ASSIGNEE(S):	Amgen Inc., Thousands Oaks, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6080845		20000627
APPLICATION INFO.:	US 1999-238182		19990128 (9)
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1998-129549, filed on 5 Aug 1998, now abandoned		
DOCUMENT TYPE:	Utility		
FILE SEGMENT:	Granted		
PRIMARY EXAMINER:	Huff, Sheela		
LEGAL REPRESENTATIVE:	Mazza, Richard J., Levy, Ron K., Odre, Steven M.		
NUMBER OF CLAIMS:	1		
EXEMPLARY CLAIM:	1		
NUMBER OF DRAWINGS:	6 Drawing Figure(s); 5 Drawing Page(s)		
LINE COUNT:	672		

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A non-naturally occurring peptide derived from EGF-like domains of NDF/**heregulin** protein isoforms is used to stimulate the **proliferation** of **cells** in the sensory epithelium of the **inner ear**. A monoclonal antibody against adult rat utricular epithelium is also described.

SUMM This invention relates to the NDF/**heregulin** protein family, and more specifically to the use of a derivative peptide to stimulate the **proliferation** of sensory epithelial **cells** of the **inner ear** for the treatment of vestibular **disorders**. The invention also relates to monoclonal antibodies raised against adult rat utricular epithelium.

SUMM . . . Letters, Volume 349, pages 139-143 (1994); and Carraway et al., Journal of Biological Chemistry, Volume 269, pages 14303-14306 (1994). The NDF/**heregulin** family is considered to also include ARIA and glial growth factor (GGF); see, respectively, Falls et al., Cell, Volume 72,. . .

SUMM The present invention comprises the use of a peptide of following sequence as a **growth** stimulant for sensory epithelial **cells** of the **inner ear**:

SUMM . . . a hybrid form derived from the EGF-like domains of NDF-.alpha. and NDF-.beta.. However, the usefulness of this molecule as a **growth** stimulant for sensory epithelial **cells** of the utricle in the **inner ear**, which is demonstrated in

the working examples below, has not been previously recognized. Because all of the vestibular organs (e.g.,. . . way to contact and treatment with the present peptide. Thus, the peptide is expected to be useful to treat vestibular **disorders** such as, for example, loss of balance due to utricular degeneration or disease in mammals, including humans. The peptide may also be useful to treat hearing loss in mammals, including humans, which is attributable to the degeneration of **inner ear** hair **cells**, i.e., by **regenerating** such hair **cells** in association with sensory epithelium.

DRWD . . . a graph comparing the mitogenic activity (as BrdU-positive nuclei) of the peptide of SEQ ID NO: 1 ("Peptide") with other NDF/**heregulin**-derived peptides on **inner ear** sensory epithelial **cells**.

DETD The mitogenic activity of the peptide of SEQ ID NO: 1 on the vestibular sensory epithelium of the mammalian **inner ear** suggests that it may also be useful to **regenerate** hair **cells**, which are critical for hearing. Thus, the peptide may be beneficial for treating hearing loss associated with deteriorated or damaged **inner ear** hair **cells**, and such applications are included within the therapeutic treatments made possible by the present invention.

DETD Sensory epithelial **cells** obtained from utricles in the **inner ear** of both seven day-old (infant) rats and six week-old (adult) rats were isolated with the use of thermolysin treatment; see. . . page 87 (1995). All edges were trimmed away and the central portion of the epithelium was cut into quarters. Epithelial **cells** from the infant rats were cultured in DMEM/F12 with 10% FBS (Gibco BRL, Grand Island, N.Y.), and 3 micrograms per. . . 1 or 50 ng/ml of recombinant derived FGF-10, recombinant derived FGF-16, recombinant derived ciliary-derived neurotrophic factor (CNTF), recombinant derived neurotrophic **growth** factor (NGF), recombinant derived glial-derived neurotrophic factor (GDNF), recombinant derived keratinocyte **growth** factor (KGF), or a control (no **growth** factor present). The experiment was ended by fixing in 4% paraformaldehyde for one hour.

DETD Using the test procedure of Example 1, the peptide of SEQ ID NO: 1 was compared with members of the NDF-**heregulin** family in primary cultures of young rat utricular sensory epithelial cells, at a treatment concentration of 50 ng/ml in each. . .

DETD **Generation** of Monoclonal Antibodies Against Sensory Epithelial **Cells** of Rodent **Inner Ear**

DETD The lack of a specific marker for sensory epithelium **cells** adds to the challenges associated with research on hair **cell regeneration** in the **inner ear** of mammals. Monoclonal antibodies against hair **cells** have been reported in the literature; Finley et al., Assoc. Res. Otolaryngol. Abstr., Volume 20, page 16 (1997) and Holley et al., J. Neurocytol., Volume 24, pages 853-864 (1997). However, none of these antibodies are specific to supporting **cells** in the mammalian vestibular organs.

DETD . . . is a description of the preparation of four distinct monoclonal antibodies raised against rat utricular epithelia which specifically label supporting **cells** of the vestibular organs in the **inner ear** of the rodent. These antibodies constitute an additional aspect of the present invention.

DETD In this method, sensory epithelia were isolated from adult rat **inner ear** utricles by the thermolysin method; see above for description. Seventy pieces of epithelia were homogenized by

ultrasound, and then emulsified. . . showed high titer against the antigen (i.e., the utricle extract). Mouse splenocytes were harvested and then fused with HL-1 myeloma **cells** (Kohler and Milstein, Nature, Volume 256, pages 495-497 (1975). Screening for monoclonal antibodies was conducted by immunostaining on frozen 10-micron. . .

DETD Each of the monoclonal antibodies specifically stained the supporting **cells**, but with a characteristically different pattern. SC-1 stained the top portion of the supporting **cells** brightly, while gradually decreasing around the **cell** nuclei. SC-2 stained only the top portion of the supporting **cells**. SC-3 immunoreactivity was concentrated on the lower cytoplasmic portion of the supporting **cells** in neotal rat utricles, and migrated to the upper portion in adult utricles. SC-4 immunoreactivity was found mostly in the supporting **cell** apex of the adult utricle. SC-4 and SC-3 immunostaining was found in embryonic progenitors of supporting **cells** of the **inner ear**.

L7 ANSWER 7 OF 7 CAPLUS COPYRIGHT 2002 ACS

AB Hair **cell** loss due to acoustic and ototoxic damage often leads to hearing and balance impairments. Although a spontaneous even in chicks and lower vertebrates, hair **cell** replacement occurs at a much lower frequency in mammals presumably due to a very low rate of supporting **cell proliferation** following injury. The authors report that **heregulin**, a member of the neuregulin family, dramatically enhances **proliferation** of supporting **cells** in postnatal rat utricular epithelial sheet cultures after gentamicin treatment, as revealed by bromo-deoxyuridine (BrdU) immunocytochem. A dose-dependent study shows that the maximal effects of **heregulin** are achieved at 3 nM. The mitogenic effects of **heregulin** are confirmed in utricular whole mount cultures. Autoradiog. of the utricular whole mount cultures shows that **heregulin** also enhances the no. of tritiated thymidine-labeled **cells** within the hair **cell** layer. TaqMan quant. RT-PCR anal. and immunocytochem. reveal that **heregulin** and its binding receptors (ErbB-2, ErbB-3 and ErbB-4) are expressed in the **inner ear** sensory epithelium. Of several ligands activating various ErbB receptors, including **heregulin**, neuregulin-3, .beta.-cellulin, heparin binding-epidermal **growth** factor (HB-EGF), transforming **growth** factor-.alpha. (TGF-.alpha.) and EGF, **heregulin** shows the most potent mitogenic effects on supporting **cells**. Because neuregulin-3 that signals only through ErbB-4 does not show an effect, these data suggest that activation of the ErbB-2-ErbB-3 heterodimeric complexes, rather than ErbB-4, is crit. for the **proliferative** response in the utricular sensory epithelium. In addn., gentamicin treatment induces an upregulation of **heregulin** mRNA. Considered together, **heregulin** may play an important role in hair **cell regeneration** following ototoxic damage.

ACCESSION NUMBER: 2000:652691 CAPLUS
DOCUMENT NUMBER: 133:345081
TITLE: **Heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage
AUTHOR(S): Zheng, J. Lisa; Frantz, Gretchen; Lewis, Annette K.; Sliwkowski, Mark; Gao, Wei-Qiang
CORPORATE SOURCE: Department of Neuroscience, Genentech Inc., South San Francisco, CA, 94080, USA
SOURCE: Journal of Neurocytology (2000), Volume Date 1999, 28(10/11), 901-912

CODEN: JNCYA2; ISSN: 0300-4864
PUBLISHER: Kluwer Academic Publishers
DOCUMENT TYPE: Journal
LANGUAGE: English
REFERENCE COUNT: 58 THERE ARE 58 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI **Heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage

AB Hair **cell** loss due to acoustic and ototoxic damage often leads to hearing and balance impairments. Although a spontaneous even in chicks and lower vertebrates, hair **cell** replacement occurs at a much lower frequency in mammals presumably due to a very low rate of supporting **cell proliferation** following injury. The authors report that **heregulin**, a member of the neuregulin family, dramatically enhances **proliferation** of supporting **cells** in postnatal rat utricular epithelial sheet cultures after gentamicin treatment, as revealed by bromo-deoxyuridine (BrdU) immunocytochem. A dose-dependent study shows that the maximal effects of **heregulin** are achieved at 3 nM. The mitogenic effects of **heregulin** are confirmed in utricular whole mount cultures. Autoradiog. of the utricular whole mount cultures shows that **heregulin** also enhances the no. of tritiated thymidine-labeled **cells** within the hair **cell** layer. TaqMan quant. RT-PCR anal. and immunocytochem. reveal that **heregulin** and its binding receptors (ErbB-2, ErbB-3 and ErbB-4) are expressed in the **inner ear** sensory epithelium. Of several ligands activating various ErbB receptors, including **heregulin**, neuregulin-3, .beta.-cellulin, heparin binding-epidermal **growth** factor (HB-EGF), transforming **growth** factor-.alpha. (TGF-.alpha.) and EGF, **heregulin** shows the most potent mitogenic effects on supporting **cells**. Because neuregulin-3 that signals only through ErbB-4 does not show an effect, these data suggest that activation of the ErbB-2-ErbB-3 heterodimeric complexes, rather than ErbB-4, is crit. for the **proliferative** response in the utricular sensory epithelium. In addn., gentamicin treatment induces an upregulation of **heregulin** mRNA. Considered together, **heregulin** may play an important role in hair **cell regeneration** following ototoxic damage.

ST **heregulin** utricular sensory epithelium regeneration proliferation ototoxic damage; hair cell proliferation regeneration ototoxic damage

IT Cell proliferation
Regeneration, animal
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-3, heterodimeric complexes with ErbB-2; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BOC (Biological occurrence); BSU (Biological study, unclassified); BIOL (Biological study); OCCU (Occurrence)
(**heregulin**, ErbB-4; **heregulin** enhances regenerative

proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin**, ErbB-4; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Growth factor receptors
RL: BOC (Biological occurrence); BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); OCCU (Occurrence); PROC (Process)
(**heregulin**, erbB-3, heterodimeric complexes with ErbB-2; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT neu (receptor)
RL: BOC (Biological occurrence); BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); OCCU (Occurrence); PROC (Process)
(heterodimeric complexes with ErbB-3; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT **Ear**
(**inner**, sensory epithelium; **heregulin** enhances **regenerative proliferation** in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Hearing
(loss; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Heregulins
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(neuregulin-3; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Ear
(organ of Corti, hair cell; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Ear
(ototoxicity; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Ear
(utriculus; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT Transforming growth factors
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(.alpha.-; **heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

IT 1403-66-3, Gentamicin
RL: ADV (Adverse effect, including toxicity); BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after gentamicin-induced ototoxic damage)

IT 62229-50-9, Epidermal growth factor 154531-34-7, Heparin-binding epidermal growth factor-like growth factor 163150-12-7, BetaCellulin
RL: BAC (Biological activity or effector, except adverse); BSU (Biological

study, unclassified); BIOL (Biological study)
(**heregulin** enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic damage)

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